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Regulation of gene expression by ractopamine in porcine skeletal muscle

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Regulation of gene expression by ractopamine in porcine skeletal muscle

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Signatures have been redacted for privacy

DEDICATION

This thesis is dedicated my parents, Marlin and Donna, who instilled in me the importance of integrity, self-motivation, and having an impeccable work ethic, traits which continue to carry me through.

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ABSTRACT

The β-adrenergic agonist, ractopamine, stimulates muscle hypertrophy and increases lean body composition in the pig. Increases in protein synthetic rate, changes in muscle fiber type, and increases in the pre-translational expression of skeletal muscle-specific genes in skeletal muscle have been documented after treatment with ractopamine. These effects may be the accumulation of changes in enzymatic activity, substrate utilization, and/or gene transcription. This research addressed ractopamine's capacity to alter gene expression. In the first study, the transcriptional responsiveness of the porcine skeletal α-actin promoter was evaluated. In porcine myotubes, ractopamine stimulated the accumulation of skeletal α-actin mRNA. However, the previously cloned full-length porcine skeletal α-actin promoter was unresponsive to ractopamine treatment. It is possible that a novel mechanism regulates the accumulation of skeletal α-actin mRNA, possibly via an unidentified regulatory element within, or adjacent to, the gene's locus. Alternatively, skeletal α-actin mRNA stability could be enhanced in response to ractopamine. In the second study, the suppression subtractive hybridization technique was used to identify differentially expressed genes in porcine skeletal muscle after feeding ractopamine to pigs for three days. From this initial screen, nine genes and one expressed sequence tag were tentatively identified. Further analysis confirmed that ractopamine increased the steady state mRNA abundance of calmodulin-1 by approximately two-fold. This is the first study to implicate calcium-modulated proteins and calcium signaling as potentially being involved in

the cellular response to ractopamine in porcine skeletal muscle. Future investigations should address the physiological significance of increased calmodulin expression, as calcium/calmodulin dependent proteins have been implicated in both muscle hypertrophy and muscle fiber type transition.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

The ability to manipulate growth and protein accretion in domestic livestock species is important to the sustainability of animal agriculture. Livestock producers constantly seek to increase the output of nutritious meat products while improving production efficiency. Historically, progress in this regard has relied on intensifying genetic selection, developing better nutrition programs, and adopting improved production systems. More recently, producers have begun to appreciate the anabolic enhancing capacity of growth promotants, such as antimicrobials, growth factor analogues, and β-adrenergic agonists. In lieu of considerable research into the efficacy and potential economic benefit, the livestock industry stands to benefit from the future integration of such compounds into modern production schemes.

When incorporated into livestock diets, β-adrenergic agonists improve lean carcass composition, dressing percentage, growth rate, and growth efficiency without adversely affecting meat quality in most species. One class of synthetic β-adrenergic agonists, the phenethanolamines, has a profound effect on skeletal muscle mass. Increased rates of protein synthesis and/or decreased rates of protein degradation are hallmark responses to phenethanolamines. A sustained increase in net protein accretion ultimately leads to skeletal muscle hypertrophy and increased muscle mass.

Our current understanding of the molecular processes stimulated by phenethanolamines, as well as other classes of β-adrenergic agonists, is limited.

Phenethanolamines, such as ractopamine and clenbuterol, are high affinity ligands for the β-adrenergic receptors, which are present on cellular membranes in skeletal muscle and other tissues. Several signal transduction pathways may mediate cellular response to β-adrenergic agonists. However, few studies have sought to directly clarify the specific mechanisms that lead to hypertrophy in treated livestock. The future development of targeted growth promotants for use in animal agriculture may benefit from the elucidation of the molecular mechanisms by which phenethanolamines initiate and maintain long-term anabolic affects in skeletal muscle. The identification of regulatory mechanisms that control gene expression in response to ractopamine would provide a framework to address this question.

This project investigated the effect of ractopamine on muscle specific gene expression in the pig. In the first study, the hypothesis that ractopamine directly regulates the expression of the porcine skeletal α-actin gene via cis-acting DNA elements was tested. The observed increase in pre-translational expression of skeletal α-actin in pigs treated with ractopamine parallels the observed increase in myofibrillar protein expression and skeletal muscle hypertrophy. Therefore, identifying regulatory mechanisms that control the expression of this gene should provide insight into the mechanisms by which ractopamine induces skeletal muscle hypertrophy. In a later study, we identified differentially expressed genes by a polymerase chain reaction (PCR) based cDNA subtraction technique known as suppression subtractive hybridization. Polyadenylated mRNA was isolated from the longissimus dorsi muscle of pigs fed ractopamine for seventy-two hours and compared to controls. Using this technique, a normalized cDNA library is generated

from mRNA transcripts and amplified by PCR. Exponentially amplified cDNA amplicons may correspond to genes regulated by ractopamine in treated skeletal muscle. Genes expressed in response to three days of ractopamine stimulation in skeletal muscle were identified by homology after comparison of the subcloned amplicon sequence to public databases. Presumably, these genes are regulated by ractopamine and are required for sustained muscle hypertrophy and/or alterations in myosin heavy chain isoform content. The cumulative findings of this project will provide direction for future studies designed to explain the molecular mechanisms by which β-adrenergic agonists enhance lean body composition.

Thesis Organization

This thesis presents the author's research in the alternative format. The first chapter contains a general introduction and a review of the relevant scientific literature. The second chapter is a manuscript prepared for submission to the Journal of Animal Science, which details the author's work that addresses the transcriptional regulation of the skeletal α-actin gene in response to ractopamine in an *in vitro* model. The third chapter is a manuscript prepared for submission to the Journal of Animal Science, which details the procedures used to putatively identify genes that are differentially expressed in response to ractopamine stimulation *in vivo*. The final chapter discuses the results obtained by the author as they pertain to our understanding of skeletal muscle hypertrophy and increased protein accretion in response to phenethanolamine treatment.

Review of Relevant Literature

Catecholamines: Nature's β-Adrenergic Receptor Agonists

Before the turn of the twentieth century, George Oliver and Edward Albert Schäfer made the seminal observation that adrenal preparations increased blood pressure (Oliver & Schafer, 1894). This established, for the first time, that the adrenal medulla was potentially responsible for hormonal action of physiological consequence. From similar preparations, Abel (1898) isolated the hormone responsible for these effects, which he referred to as epinephrine. Cannon and colleagues characterized many of the biological actions of epinephrine (Cannon, 1940), and coined the phrase "fight or flight hormone" to describe the ability of epinephrine to maintain physiological homeostasis during stress. A second hormone, norepinephrine, which is nearly indistinguishable from epinephrine, was determined to be of medullary origin (Goldenberg et al., 1949; von Euler & Hamberg, 1949). Therefore, Oliver, Schäfer, Cannon, and others demonstrated not only the individual action of epinephrine, but also the combined synergistic affects of epinephrine and norepinephrine.

Epinephrine and norepinephrine, structurally classified as catecholamines, are the principle hormones of the autonomic nervous system and mediate a diverse array of physiological processes. Norepinephrine is the major neurotransmitter in the sympathetic nervous system and is the more abundant catecholamine in the peripheral circulation. Both norepinephrine and epinephrine act as hormones when secreted by the adrenal medulla (Ganong, 2001). In response to stress, epinephrine initiates catabolism of body energy reserves through increased rates of liver

glycogenolysis, white adipose lipolysis, and brown adipose thermogenesis. The catecholamines support skeletal and cardiac muscle hypertrophy under some physiological conditions, but this anabolic affect is limited. In addition, cardiac contractility, vasoconstriction, bronchodilation, and gastric reflexes are physiological processes regulated by norepinephrine and epinephrine. While this is not an all-inclusive list, the diverse mechanisms activated in response to catecholamine release appear to be of evolutionary significance, as they are highly conserved across species.

Adrenergic Receptors and Signal Transduction

Cellular responses to norepinephrine and epinephrine are initiated when they bind to two distinct classes of receptors: the α- and β-adrenergic receptors. Both of these catecholamines signal through adrenergic receptors, albeit with different affinities. Norepinephrine has greater affinity for the α-adrenergic receptors, whereas epinephrine has higher affinity for the β-adrenergic receptors (Ganong, 2001). Assigned initially by pharmacological properties and, later, by identification of different encoding genes and protein products, the α-adrenergic receptors are currently divided into nine subtypes (α1A, α1B, α1D, α1L, α2A, α2B, α2C, α2D, and α2A/D) (Civantos & Aleixandre de Artinano, 2001); only three subtypes (β1, β2, and β3) comprise the β-adrenergic receptor family (Wallukat, 2002).

The adrenergic receptors belong to a larger class of structurally similar seven transmembrane-domain proteins, or G-protein coupled receptors. As the name implies, these receptors couple to heterotrimeric guanine nucleotide exchange

proteins (G-proteins) that serve as signal transducers to activate or inhibit other proteins. The assortment of physiological responses to catecholamines is a net result of the combined diversity of the adrenergic receptors, their multiple associated G-proteins, and proteins regulated by G-proteins through second messenger molecules, such as cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP_3) (Pierce et al., 2002). Ultimately, these second messengers regulate effector proteins involved in ion transport, gene transcription, and signal transduction cascades, which adds to the complexity of the cellular response to ligand activation of the membrane-associated receptor. Consequently, our knowledge of the molecular mechanisms regulated by this class of receptors has yet to be exhausted.

Catecholamine Regulation of Adipose Metabolism

In all mammals, white adipose tissue is the major storage site of neutral fat (Ganong, 2001). Adipose tissue hypertrophies when energy exceeds metabolic demand. Excess dietary fatty acids and glucose are stored in adipocytes as triglycerides through the biochemical process of lipogenesis. In a fed state, lipogenesis is predominately regulated by insulin, which is produced in pancreatic B cells and stimulates glucose uptake. During fasting, lipolysis causes atrophy, or regression, of adipose tissue. Lipases catalyze the breakdown of triglycerides to glycerol and fatty acids, which then are available for β-oxidation and ATP synthesis in peripheral tissues. The catecholamines regulate both lipolysis and lipogenesis via distinct and well-defined mechanisms.

Catecholamine-induced lipolysis represents a classical signal transduction pathway regulated by catecholamines. While all three β-adrenergic receptor subtypes activate this pathway, the β_3-adrenergic receptor may be the most efficacious in adipose because this subtype is more resistant to desensitization (Carpene et al., 1998). Epinephrine binds to the β-adrenergic receptors on adipocyte membranes and induces a conformational change in the receptor. This allows for the dissociation of the associated heterotrimeric G-proteins. The G_α subunit exchanges guanosine monophosphate (GMP) for guanosine tri-phosphate (GTP), dissociates from the $G_\beta G_\gamma$ heterodimer, and translocates to interact with adenylyl cyclase. The G_α subunit may stimulate (G_s) or inhibit (G_i) adenylyl cyclase activity. Adenylyl cyclase, which is a membrane bound enzyme, catalyzes the conversion of adenosine tri-phosphate (ATP) to cyclic adenosine monophosphate (cAMP). Increased intracellular cAMP levels activate cAMP-dependent protein kinase A (PKA), which in turn phosphorylates and activates hormone-sensitive lipase. Activated lipase releases free fatty acids into the bloodstream. Glucagon, produced by the pancreas, stimulates hormone-sensitive lipase in the same manner through another G-coupled protein receptor, the glucagon receptor (Jiang & Zhang, 2003).

Catecholamine-mediated antagonism of insulin action further intensifies adipose catabolism by lowering plasma insulin levels and by reducing glucose uptake by adipose tissue. Through the activation of the abundant α_2-adrenergic receptor population on B cells, catecholamines indirectly regulate lipogenesis in

adipose tissue (Filipponi et al., 1986). The α_2-adrenergic receptor associates with the G_i subunit, which lowers intracellular cAMP and inhibits voltage dependent calcium channels. This decreased intracellular calcium influx disrupts the exocytosis of insulin from pancreatic B cells (Hsu et al., 1991). In adipose, catecholamines diminish the effects of insulin directly. Activation of β_3-adrenergic receptors stimulates the cAMP-PKA pathway. This attenuates insulin-induced glucose uptake via the inhibition of insulin-sensitive glucose transporter (GLUT4) translocation to the cellular membrane (Carpene et al., 1993; Yen et al., 1998). Additionally, GLUT4 transcription is selectively repressed by increased cAMP concentrations in cultured adipocytes (Kaestner et al., 1991), which provides a long-term interruption of glucose uptake and slows adipose hypertrophy.

Catecholamine Regulation of Muscle Protein Turnover

In order for skeletal muscle to hypertrophy, myofibrils, which are the highly ordered protein networks responsible for muscle contraction and shape, increase in size and number within the skeletal muscle cell, or myofiber. The rate of protein synthesis must exceed protein degradation for hypertrophy to occur, as newly synthesized myofibrils add to existing myofibrils to increase cell size. These processes are highly regulated by nutritional, hormonal, and mechanical stimuli. Even though catecholamines are predominantly associated with adipose catabolism, both epinephrine and norepinephrine modify the anabolic processes associated with muscle hypertrophy under certain physiological conditions.

The inhibition of catecholamine action by adrenal ablation increases proteolysis by 20% in rat skeletal muscle (Navegantes et al., 2002). Epinephrine

activates the cAMP-PKA pathway via the β_2-adrenergic receptor and selectively inhibits calcium-dependent proteolysis in rat skeletal muscle (Navegantes et al., 2000). Calcium-dependent proteolysis is mediated by the activation of the thiol proteases, μ-, m-, and p94 calpain, and the activity of a specific inhibitor, calpastatin (Goll et al., 1992). The calpains cleave myofibrillar proteins that comprise the myofibril into smaller peptides. The selective inhibition of the calpains, therefore, supports muscle mass and has been implicated in muscle growth (Huang & Forsberg, 1998). Protein kinase A modifies calpastatin activity, which in turn alters protease activity (Cong et al., 1998), and may inhibit m-calpain activity (Shiraha et al., 2002; Smith et al., 2003). Both affects require phosphorylation of the target protein by PKA. Through this mechanism, epinephrine supports increased skeletal muscle size predominantly through the inactivation of proteolytic processes in the rat.

In cardiac muscle, the cAMP-PKA pathway regulates contractility in response to the activation of β-adrenergic receptors by epinephrine (Wallukat, 2002), but other catecholamine-initiated pathways appear to contribute to cardiac hypertrophy (Scheuer, 1999). Norepinephrine binds to α-adrenergic receptors, which stimulates the mitogen activated protein kinase (MAPK) pathway (Gillespie-Brown et al., 1995) and increases protein synthesis (Ponicke et al., 2001; Xiao et al., 2001; Xin et al., 1997). In particular, the expression of hypertrophic markers, such as skeletal α-actin and α- and β-myosin heavy chain, increases in cardiomyocytes treated with norepinephrine (Bishopric & Kedes, 1991; Bishopric et al., 1987; Long et al., 1991).

The α-adrenergic activation of the MAPK pathway requires association of a G_q-dependent pathway (Naor et al., 2000). The guanine exchange protein G_q selectively activates phospholipase C, which cleaves membrane phospholipids into the second messengers diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP_3). Inositol 1,4,5-triphosphate increases intracellular calcium release from the endoplasmic reticulum and free calcium stimulates calmodulin kinase II (CaMK II) activity, which activates the MAPK pathway through the activation of Ras protein. The β-adrenergic receptors may also activate MAPK, although this appears to require both G_s activation of the cAMP-PKA pathway and G_i activation of Src/Ras signaling pathways (Yamazaki & Yazaki, 2000). The ability of catecholamines to induce protein synthesis in skeletal muscle may follow similar mechanisms, but considerably more work has been done in cardiac muscle, as catecholamines have been implicated in the progression of pressure overload myocardial hypertrophy (Rapacciuolo et al., 2001).

β-Adrenergic Receptor Desensitization

The prolonged exposure to catecholamines may be detrimental to the target cell. Receptor desensitization is one mechanism by which the body protects itself against cellular damage. The β_1- and β_2- adrenergic receptor subtypes, in a ligand bound state, are susceptible to phosphorylation by both PKA and β-adrenergic receptor kinase (βARK) family members (Strosberg, 1995). Upon dissociation of the heterotrimeric G-proteins, the $G_\beta G_\gamma$ subunits recruit βARK to the ligand bound receptor while prolonged accumulation of cAMP mediates PKA localization to the

receptor. The ensuing phosphorylation event stimulates endocyosis of the β-adrenergic receptor through a clathrin-coated vesicle, a process referred to as homologous receptor desensitization (Luttrell & Lefkowitz, 2002). Receptor endocytosis leads to either recycling to the membrane or, more commonly, lysosomal degradation of the ligand-bound receptor. Interestingly, the β_3-adrenergic receptor is resistant to phosphorylation by PKA and βARK family members, as this subtype lacks targeted phosphorylation domains (Strosberg, 1995). While this is not the only mechanism known to attenuate β-adrenergic responsiveness in the target cell, it is the most common and provides the most direct feedback inhibition.

Synthetic Adrenergic Receptor Agonists

As the study of epinephrine and norepinephrine action through their respective receptors evolved, so did the desire to design synthetic structural analogues capable of activating specific adrenergic receptor subtypes for both research and clinical applications. Today, adrenergic receptor agonists and antagonists are effective clinical treatments for shock, hypotension, hypertension, cardiac arrhythmias, congestive heart failure, nasal decongestion, asthma, weight reduction, narcolepsy, and attention-deficit hyperactivity disorder (ADHD) (Hoffman, 2001).

β-Adrenergic Receptor Agonists in Livestock Production

Early work with epinephrine indicated that it increases adipose catabolism and that this effect mediated by activation of the β-adrenergic receptors. In livestock, such a response would be beneficial to the production of lean meat

products. Beginning in 1984, several studies demonstrated that β-adrenergic agonists were effective modifiers of body composition in livestock species. One of the first reports demonstrated that clenbuterol not only decreased fat deposition, but also surprisingly increased total body protein content in broiler chickens (Dalrymple et al., 1984). Similar reports soon followed. In the pig, clenbuterol (Jones et al., 1985), cimaterol (Mersmann et al., 1987), salbutamol (Warriss et al., 1990b), and ractopamine (Watkins et al., 1990) all decreased carcass fat and increased muscle volume. Sheep (Kim et al., 1987), steers (Moloney et al., 1990), mice (Rothwell & Stock, 1985), rats (Reeds et al., 1988), and quail (Merkley & Garwood, 1989) all respond positively to various β-adrenergic agonists, as evidenced by increased lean body composition. The unexpected improvement in carcass protein content stimulated further research into the efficacy and potential economic benefit of these compounds to enhance efficient meat production.

Ractopamine

Although efficacy and toxicological studies have since identified a number of effective β-adrenergic agonists, ractopamine is currently the only commercially available agonist approved by the United States Food and Drug Administration (FDA) for use in livestock. Producers may feed this orally active β-adrenergic agonist to swine from 68 to 109 kilograms of body weight with no pre-slaughter withdrawal time (FDA, 2002). Even though other β-adrenergic agonists may become legally available to producers in the future, the remainder of this review will focus on the biological response to ractopamine in swine.

Ractopamine: Growth and Body Composition

Watkins et al. (1990) reported that ractopamine improved the rate of average daily gain and feed utilization (feed:gain) in the pig. Carcass composition also improved significantly. Carcass dressing percentage, longissimus muscle area, dissected carcass lean, and estimated fat-free muscle percentage increased in a dose dependent manner, while the undesirable carcass traits of carcass fat depth, average backfat, and dissected fat decreased after feeding ractopamine. Others have reported similar findings (Moody et al., 2000; Stites et al., 1991; Wray-Cahen et al., 1998). These responses are consistent regardless of sex (Dunshea et al., 1993b; Uttaro et al., 1993; Williams et al., 1994), genotype (Bark et al., 1992; Gu et al., 1991; Yen et al., 1990; Yen et al., 1991), and final slaughter weight (Crome et al., 1996) of the ractopamine treated pigs.

Ractopamine: Affects on Meat Quality

In order for β-adrenergic agonists to truly make an impact in livestock production systems, they must have no adverse effects on product quality. Reports addressing the effects of ractopamine on pork quality are often contradictory and inconsistent. Some studies have found that ractopamine does not adversely affect quality traits (Crome et al., 1996; Watkins et al., 1990) while others suggest that pork from treated pigs is paler in color, tougher, and firmer (Uttaro et al., 1993), qualities that are not appealing to consumers. Marbling and water holding capacity are unaffected in most trials (Crome et al., 1996; Uttaro et al., 1993). However, salbutamol adversely affects pork color (Warriss et al., 1990a; Warriss et al., 1990b) and other β-adernergic agonists appear to adversely affect meat tenderness in

ruminant species (Bardsley et al., 1992; Parr et al., 1992; Pringle et al., 1994; Speck et al., 1993). It is unclear why responses differ across species or why certain β-adrenergic agonists are more detrimental to product quality than others are. Differences in dose, treatment duration, and genetic backgrounds of the tested animals, as well as sampling techniques, could account for the discrepancies between these studies. Nonetheless, the response to β-adrenergic agonists deserves more consideration by meat scientists in order to ensure that the incorporation of these compounds into modern production systems is not detrimental to product quality.

Ractopamine: Potential Nutritional Constraints

While ractopamine can be effectively used to manipulate growth and composition, there may be room for further adjustment to design truly cost-effective nutrition programs that maximize biological response. An interaction appears to exist between ractopamine treatment and nutrient density of the diet. Increased dietary crude protein (Adeola et al., 1990; Dunshea et al., 1993a; Xiao et al., 1999), energy density (Williams et al., 1994), or both (Mitchell et al., 1991) may be required to elicit a maximal response. This is apparently due to the fact that animals treated with β-adrenergic agonists tend to consume less feed per unit of body mass and have enhanced muscle growth, which may require increased crude protein input and/or optimized amino acid distribution in the diet (Reeds & Mersmann, 1991). The concept of limited performance through the restriction of dietary protein is particularly evident in genotypes with a high capacity for lean gain (Bark et al., 1992). Additionally, trends observed in one study indicate that the amino acid composition

of longissimus dorsi muscle may change in response to ractopamine (Xiao et al., 1999). Nutritional studies conducted more recently in the pig have been designed to optimize dietary energy density and protein content in order to maximize the response to ractopamine in modern swine genotypes (Trapp et al., 2002; Weber et al., 2002; Webster et al., 2002).

Ractopamine as a Repartitioning Agent

Hammond (1952) proposed that the manipulation of physiological parameters that regulate nutrient utilization in livestock species is the most efficacious means to maximize production and achieve a desired phenotype. In this model, evolutionary mechanisms direct the utilization of absorbed nutrients preferentially along a tissue hierarchy that supports the maturation of vital tissues first (brain, central nervous system, and gastrointestinal tract), followed by structural tissue (the skeleton and skeletal muscle), and finally reserve deposits (fat). The observed changes in body composition and growth rate, as well as the divergence of tissue deposition from more established patterns of development, suggest that ractopamine alters the coordinated regulation of metabolism to support and enhance lean growth. However, sufficient detail, particularly at the cellular level, as to how this is accomplished remains largely elusive (Eisemann, 1994).

β-Adrenergic Receptors in Porcine Tissue

The coding sequences for all three β-adrenergic receptor subtypes expressed in pig tissues share a high level of homology with other mammalian species (Cao et al., 1998; Liang et al., 1997; Smith et al., 2001). While heterogeneously expressed, the β_1-adrenergic receptor is the predominant subtype in most porcine tissues

(McNeel & Mersmann, 1999). Quantification of mRNA abundance by reverse transcription revealed unequal transcript distribution in porcine heart (72% β_1: 28% β_2: 0.25% β_3), skeletal muscle (60%: 39%: 0.7%), subcutaneous adipose (73%: 20%: 7%), lung (67%: 33%: 0.2%), and liver (45%: 55%: 0.0%). These values are in agreement with competitive displacement assays (Liang & Mills, 2002). Based on these findings and limited kinetic studies (Spurlock et al., 1993), ractopamine was designated as a specific agonist for the β_1-adrenergic receptor subtype (Moody et al., 2000). In contrast, other β-adrenergic agonists capable of modifying body composition, such as clenbuterol, are specific for the β_2-adrenergic receptor subtype (Mersmann, 1998).

Effect of Ractopamine on Adipose in the Pig

Peterla and Scanes (1990) treated excised porcine adipose tissue from market weight pigs with ractopamine *in vitro*. By monitoring glycerol and free fatty acid release, as well as the incorporation of carbon-14 labeled glucose into fatty acids, they determined that ractopamine stimulated lipolysis and slowed the rate lipogenesis similar to epinephrine. When reduced to the cellular level, ractopamine increased cAMP accumulation (Mills et al., 2003) and lipolysis (Liu et al., 1989; Mills et al., 2003; Weber et al., 1992), antagonized insulin action (Dubrovin et al., 1990; Liu & Mills, 1990; Mills et al., 1990), and decreased fatty acid synthase activity (Weber et al., 1992) in adipocytes. These observations are consistent with increased lipid catabolism via activation of β-adrenergic receptors. Recently, Mills et al. (2003) demonstrated that ractopamine was equally efficacious at activating

lipolysis and cAMP accumulation via both cloned β_1- and β_2-adrenergic receptors, which the investigators over expressed in porcine adipocytes. However, investigations into the effects of ractopamine *in vivo* provide sufficient evidence that these effects are transient and probably do not contribute to long-term fat reduction and manipulation of body composition.

In unpublished data discussed by Liu et al. (1994a), the inclusion of ractopamine for one feeding interval increased plasma concentration of non-esterified fatty acids within one hour in the pig, but this lipolytic response declined with subsequent feedings and plasma concentrations returned to basal levels within five days. Furthermore, acetyl-CoA carboxylase expression and activity did not change in fat depots isolated from ractopamine fed pigs, which signifies the lack of commitment to sustainable decreases in enzymatic activity and subsequent rates of lipogenesis (Liu et al., 1994a). Interestingly, this same group reported that acetyl-CoA carboxylase expression and activity decreased in porcine adipose after treatment with recombinant porcine somatotropin, or growth hormone, (Liu et al., 1994b), which stimulates the somatotropic axis to modify body composition in the pig.

Using an approach that combined *in vivo* treatment with *in vitro* analysis, Mills et al. (1990) removed subcutaneous fat from ractopamine fed pigs and measured lipolysis, fatty acid sythesis, malic enzyme activity, and insulin action in adipose and isolated adipocytes. When adipocytes where isolated from ractopamine fed pigs, *in vivo* lipolysis could only be stimulated to 40% of control levels by additional ractopamine treatment. Further, insulin binding in adipose tissue samples was

unaffected by feeding ractopamine. These results suggest that the ractopamine-responsiveness is diminished over the duration of treatment in adipose tissue in an insulin-independent manner.

As β-adrenergic receptor number decreases in porcine tissue over the course of treatment (Sainz et al., 1993; Spurlock et al., 1994), ractopamine most likely provokes homologous receptor desensitization. Disagreement with regard to the action of ractopamine on adipose metabolism may be a result of this phenomenon. It is likely that *in vitro* models fail to recapitulate a long-term response to ractopamine but provide a true indication of the early metabolic response (increased lipolysis, cAMP accumulation, and antagonized insulin action). The lack of committed gene expression and up-regulation of lipolytic enzymes indicates that prolonged exposure to ractopamine may establish a feedback inhibition, which attenuates β-adrenergic receptor signaling and subsequent changes in metabolism *in vivo*. The long-term regulation of fat deposition may not be a direct effect of ractopamine on fat depots but rather an indirect effect on nutrient sequestration by other more metabolically active tissues.

Effect of Ractopamine on Skeletal Muscle in the Pig

β-Adrenergic agonists increase muscle mass *in vivo* (Beermann, 2002; Kim & Sainz, 1992). Increased cell number, increased fiber length, or increased fiber area are three mechanisms known to increase skeletal muscle mass (Pearson, 1990). As increased cell number, or hyperplasia, is restricted to prenatal and early postnatal development in the pig, ractopamine increases muscle mass by regulating the latter

two mechanisms. However, sufficient detail as to how this is accomplished has remained elusive.

Ractopamine and Protein Accretion

In order for muscle hypertrophy to occure, the myofiber increases in size by the addition of new sarcomeres (length) to the existing myofibrils and/or by the addition of new myofibrils (diameter) to the myofiber. This requires increased protein expression in the myofiber. In the pig, ractopamine positively augments net protein accretion, as fractional protein synthesis increases (Adeola et al., 1992; Adeola et al., 1990; Bergen et al., 1989) with no apparent change in proteolysis (Bergen et al., 1989; Sainz et al., 1993). Adeola et al. (1992) further demonstrated that ractopamine increased the fractional synthesis rates for myofibrillar proteins relative to sarcoplasmic protein synthesis in the longissimus dorsi and biceps femoris muscles. Additionally, ractopamine increased myofibrillar protein synthesis in both rat (Anderson et al., 1990) and C2C12 mouse myotubes (Shappell et al., 2000) in cell culture.

Skeletal α-actin is the predominant actin isoform present in the myofibrillar fraction of mature striated muscle (Bandman, 1992) and mRNA abundance increases in skeletal muscle during rapid postnatal growth in pigs (Chang et al., 1994). Therefore, increased steady state mRNA levels of skeletal α-actin indicate a pre-translational commitment to hypertrophy. Ractopamine stimulates increased skeletal α-actin mRNA abundance in pigs (Grant et al., 1993; Helferich et al., 1990; Liu et al., 1994a), as well as in steers (Smith et al., 1989), without a general

enhancement of muscle mRNA abundance. This is also true for rat skeletal muscle after cimaterol treatment (Kim et al., 1997).

Skeletal muscle is comprised of a heterogeneous population of individual myofibers. Each myofiber has distinct contractile and metabolic properties (Aberle et al., 2001) as well as myosin heavy chain (MHC) isoform expression patterns within the sarcomeres (Gerrard & Grant, 2003). Slow contracting type I and IIA fibers express the MHC isoforms I and IIA, respectively, and utilize oxidative metabolic pathways. Fast contracting type IIB fibers express predominantly MHC IIB and are glycolytic. An intermediate fiber type, which express MHC IIX, are glycolytic-oxidative (type IIX(D)).

Evidence suggests that β-adrenergic agonists also regulate the expression of the myosin heavy chain isoforms. In pigs fed ractopamine, the relative proportion of type I, type IIA, and type IIX(D) fibers decreases while the proportion of type IIB fibers increases in several muscles (Depreux et al., 2002). Though neither study differentiated between the MHC isoforms, ractopamine stimulated the *in vitro* rate of MHC synthesis in C2C12 (Shappell et al., 2000) and rat myotubes (Anderson et al., 1990). Myosin heavy chain mRNA abundance was also elevated in cimaterol treated chicken myotubes (Young et al., 1990).

Satellite Cell Proliferation

The Cheek hypothesis suggests that each myonuclei within a multinucleated myofiber controls a finite cytoplasmic volume (Cheek et al., 1971). Therefore, as the myofiber hypertrophies, DNA content of the cell must increase to support gene transcription and subsequent protein synthesis. Mitotically competent satellite cells

proliferate, differentiate, and fuse to the existing myofiber to match this demand (Allen & Rankin, 1990).

Ractopamine failed to significantly increase satellite cell proliferation in porcine cultures as the number of myotube nuclei (Grant et al., 1990b) and DNA content (Cook et al., 1995) were unchanged after treatment. Further, one *in vivo* study noted that DNA content in porcine longissimus dorsi muscle did not change after administration of ractopamine (Grant et al., 1993). While ractopamine's influence on satellite cell proliferation *in vivo* remains unexplored, the combined results of these studies indicate that it is unlikely that ractopamine directly regulates satellite cell proliferation in the pig. Even as it is likely that increased protein synthetic demand in porcine muscle would require later incorporation of satellite cells into the growing myofiber, the initial stimulation of satellite cell proliferation does not appear to be a prerequisite for ractopamine-induced hypertrophy. However, in both chicken (Grant et al., 1990a) and C2C12 mouse (Shappell et al., 2000) myoblast cultures, ractopamine increased myotube nuclei and culture DNA content, respectively. The apparent species difference has not been resolved, but variations in the expression of β-adrenergic receptor subtypes exist across species (Izevbigie & Bergen, 1996b; Young et al., 2000) and may hinder the extrapolation of these findings to the pig.

Signal Transduction

In immature skeletal muscle, cAMP accumulates in differentiated myotubes after ractopamine treatment (Cook et al., 1995; Izevbigie & Bergen, 1996a; Izevbigie & Bergen, 1996b; Shappell et al., 2000). Other β-adrenergic agonists also increase

cAMP levels in rat skeletal muscle (Kim et al., 1997). A causal association between this increase in second messenger level and increased protein synthesis in response to ractopamine remains speculative (Beermann, 2002; Mersmann, 1998; Mills, 2002; Moody et al., 2000). As porcine β-adrenergic receptors appear to be susceptible to homologous receptor desensitization in response to ractopamine treatment (Sainz et al., 1993; Spurlock et al., 1994), cAMP accumulation in porcine skeletal muscle is probably transient and rapidly attenuated. However, the increased rates of protein synthesis (Adeola et al., 1992; Adeola et al., 1990; Bergen et al., 1989) and expression of myofibrillar specific proteins (Depreux et al., 2002; Grant et al., 1993; Helferich et al., 1990) persists over an extended time course of treatment. This implies that other molecular mechanisms may be responsible for, or involved in, ractopamine-induced skeletal muscle hypertrophy.

Aims of This Study

As outlined above, prolonged β-adrenergic stimulation by administration of synthetic β-adrenergic receptor agonists induces favorable changes in body composition and improves growth efficiency in the pig. These effects appear to be the culmination of changes of many biological processes, which includes enzymatic activity, substrate utilization, and gene expression. Sufficient detail as to how this is accomplished has remained elusive, even though this question has been a focus of animal scientists for the last quarter of a century.

Modified gene expression accompanies phenotypic changes when skeletal muscle undergoes both hypertrophy (Carson, 1997; Goldspink, 2002) and atrophy

(Bey et al., 2003; Wittwer et al., 2002). While not a requirement, sustained increases in transcription of proteins involved in ion transport, biochemical pathways, cellular integrity, and signal transduction pathways tend to indicate cellular adaptations to stimuli that promote hypertrophy. For those interested in the mechanisms that control gene regulation, the initial identification of genes regulated by trophic or mechanical factors, for instance, provides a framework from which to develop innovative hypothesis-driven research, as this approach often incriminates novel signal transduction pathways and molecular events that are not fully appreciated.

The aims of this study were to 1) determine the mechanism by which ractopamine regulates the expression of the porcine skeletal α-actin gene and 2) to identify genes that are potentially regulated by ractopamine in porcine skeletal muscle. While ambitious, this study promises to provide a foundation for the further identification of ractopamine-induced regulatory mechanisms that promote increased protein accretion, muscle fiber type plasticity, and enhanced lean body composition in swine.

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CHAPTER 2. TRANSCRIPTIONAL RESPONSIVENESS OF PORCINE SKELETAL α-ACTIN TO RACTOPAMINE IN PORCINE MYOTUBES

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Abstract

Increased expression of skeletal α-actin (Sk-α-Act) parallels increased myofibrillar protein expression and muscle hypertrophy in pigs fed ractopamine. Porcine satellite cell cultures were established to determine if ractopamine directly regulates the expression of the Sk-α-Act gene via known cis-acting DNA elements. Differentiated myotubes were treated with ractopamine (10 μ*M*) in serum-free media and Sk-α-Act mRNA was quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR). After 72 h of treatment, Sk-α-Act mRNA abundance increased six-fold relative to untreated control cultures. Total protein concentrations in ractopamine treated cultures tended to be higher after 72 h ($P = 0.07$). Luciferase expression plasmids containing 5' regulatory regions of the porcine Sk-α-Act gene were transiently transfected into porcine satellite cell cultures and cells were stimulated to differentiate into myotubes. Ractopamine (10 μ*M*) was added to

serum-free media and cell lysates were assayed for luciferase activity. Ractopamine failed to increase transcriptional activity of the cloned full-length Sk-α-Act promoter (DNA sequences -1929 to +243 bp relative to the transcriptional start site), but basal activity increased over a 72-h time course ($P < 0.001$). Further, luciferase expression assays with truncated promoter constructs failed to identify a ractopamine-responsive DNA element in the cloned Sk-α-Act promoter. Our results indicate that the known cis-acting DNA elements previously described 5' of the porcine skeletal α-actin encoding gene do not confer transcriptional responsiveness to ractopamine in porcine skeletal muscle despite observed increases in Sk-α-Act mRNA abundance *in vitro* after ractopamine stimulation.

Keywords: ractopamine, pig, skeletal α-actin, expression, transcription

Introduction

Ractopamine and other β-adrenergic agonists stimulate muscle hypertrophy *in vivo* (Beermann, 2002; Kim & Sainz, 1992). In porcine skeletal muscle, ractopamine positively augments net protein accretion, as fractional protein synthesis increases with no apparent change in proteolysis (Adeola et al., 1992; Adeola et al., 1990; Bergen et al., 1989; Sainz et al., 1993). Specifically, ractopamine increases the fractional synthetic rate of myofibrillar proteins in the longissimus dorsi and biceps femoris muscles (Adeola et al., 1992). *In vitro*, ractopamine increases myofibrillar protein synthesis in both rat (Anderson et al.,

1990) and C2C12 mouse myotubes (Shappell et al., 2000). These results, and evidence that ractopamine selectively activates the β-adrenergic receptor population on skeletal muscle membranes (Spurlock et al., 1994), indicate that ractopamine has a direct effect on anabolic processes that ultimately lead to skeletal muscle hypertrophy.

Skeletal α-actin (Sk-α-Act) is the predominant actin isoform present in the myofibrillar fraction of mature striated muscle (Bandman, 1992) and Sk-α-Act expression increases in skeletal muscle during rapid postnatal growth in pigs (Chang et al., 1994). Skeletal α-actin mRNA abundance in pigs (Grant et al., 1993; Helferich et al., 1990; Liu et al., 1994) and steers (Smith et al., 1989) increases in response to ractopamine without a general increase in total muscle mRNA. These results indicate that ractopamine may stimulate skeletal muscle hypertrophy and protein accretion through the expressional regulation of muscle specific genes. However, the molecular mechanisms by which ractopamine regulates SK-α-Act expression pre-translationally have not been identified. We hypothesized that the increase in Sk-α-Act mRNA was regulated at the level of transcription.

Materials and methods

Primary Cell Culture

Porcine satellite cells were isolated from three-week-old gilts as described (Blanton et al., 2000; Doumit & Merkel, 1992). All animal handling procedures were approved by the Iowa State University Committee on Animal Care. Hindlimb muscles

(180 to 200 g) were surgically removed and mechanically dissociated in a sterilized meat grinder. Satellite cells were further isolated by digestion with bacterial protease (Type XIV at 1.2 mg/mL in phosphate buffered saline (PBS); Sigma Chemical Co., St. Louis, MO) for 40 min at 37°C and differential centrifugation. Satellite cells were then suspended in a growth media (GM) that contained minimum essential media (MEM; Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine calf serum (FBS; Atlanta Biologicals, Norcross, GA), L-glutamine (250 μg/mL; Sigma), penicillin (100 U/mL; Sigma), streptomycin (100 μg/mL; Sigma), fungizone (0.12 μg/mL; Invitrogen Corporation, Carlsbad, CA), and gentamicin (10 μg/mL; Invitrogen). Cells were plated at an initial density of 125 mg/cm^2 on 35-mm cell culture dishes coated with Matrigel (Becton Dickinson, Bedford, MA) and cultured in a 5% CO_2 humidified incubator set to 37°C. After an attachment period of 24 to 36 h, cultures were rinsed in MEM to remove residual debris and GM was replenished every 48 h thereafter unless otherwise noted.

Satellite Differentiation and Addition of Treatment Media

At 85 to 90% confluence, satellite cells were stimulated to differentiate into myotubes for 36 h by the addition of differentiation media (2% DM), which contained MEM, 2% horse serum (Fisher), L-glutamine (250 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and insulin (10^{-7} *M*; Sigma). Cells were rinsed with MEM before addition of treatment media. Treatment media consisted of Ractopamine (10 μ*M* in H_2O; Lilly Research Labs, Greenfield, IN) or volume equivalent vehicle control (H_2O) in serum-free differentiation media (SFDM) modified from that previously

described for porcine satellite cells (Doumit et al., 1996). Serum-free media contained MEM and MCDB-110 (Sigma) in a 4:1 ratio, bovine transferrin (100 μg/mL; Sigma), bovine serum albumin (0.5 mg/mL BSA; Fisher), insulin (10 μ*M*), linoleic acid (0.5 μg/mL; Sigma), and dexamethasone (25 μ*M*; Calbiochem, San Diego, CA). Treatment media was replenished every 24 h.

Total RNA Isolation from Porcine Cultures

After 72 h in treatment media, total RNA was harvested with TRIzol reagent (Invitrogen) as described by the manufacturer's protocol. Three representative cultures were collected, pooled, and processed. Total RNA was suspended in 15 μL of RNAse-free water and incubated with amplification grade Rnase-free DNase I (1 U; Invitrogen) for 15 min at room temperature to remove residual genomic DNA contamination. DNase I was inactivated by the addition of 25 m*M* EDTA and incubation at 65°C for 10 min.

First-strand cDNA synthesis

First-strand cDNA was synthesized in a 20 μL reaction which contained 1.0 μg of total RNA, 10 m*M* dNTP mix (10 m*M* each dATP, dCTP, dGTP, and dTTP), 100 m*M* 1,4-Dithio-DL-threitol (DTT), 15 U ANTI-Rnase (Ambion, Inc., Austin, TX), 50 ng oligo $(dT)_{10\text{-}20}$ primer (Invitrogen), 4 μL of 5X reaction buffer, SuperScript II $Rnase^-$ H reverse transcriptase (200 U; Invitrogen), and diethyl pyro-carbonate (DEPC) treated H_2O to volume. The reaction was performed at 42°C for 50 min. Synthesized cDNA was diluted to the equivalent of 25 ng total RNA per 1 μL of cDNA and stored at –20°C.

Quantitative Detection of mRNA Species

Quantitative PCR analysis was conducted with a Smart Cycler (Cepheid, Sunnyvale, CA) and SYBR Green I dye (Applied Biosystems, Foster City, CA) in order to determine the relative expression levels of Sk-α-Act and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in primary porcine myotubes. The concentration of double-stranded DNA after each cycle of amplification was monitored by SYBR Green I fluorescence. Each PCR reaction contained 300 n*M* (each) of gene specific primers, 10 μL of 2.5X SYBR Green Master Mix, 0.5 μL of cDNA, and sterile water to a final volume of 25 μL. After denaturation (95°C for 10 min), forty cycles of PCR were performed (95°C for 15 s, primer specific annealing temperature for 30 s, 72°C for 30 s, and fluorescence acquisition at a product-specific temperature for 10 s). Fluorescence data was captured by Smart Cycler optics after the last step of each cycle to minimize the capture of non-specific (primer dimer) product data. For Sk-α-Act, a 193-base pair (bp) amplicon was produced from forward (5'-GAG GCG AAC TAC TCC GTG TG-3') and reverse (5'-GTC AGC CAC TGC ACT TGA GC-3') primer pairs designed to amplify the seventh exon and 3'UTR of the known encoding gene (4263-4455 bp of GenBank accession number U16368). A second set of primers, forward (5'-TCC CTG CTT CTA CTG GTG CT-3') and reverse (5'-CAC AAC CTG GTG CTC AGT GT-3'), was designed to amplify a 227-bp fragment of G3PDH (955-1182 bp of AF017079). Annealing temperature for Sk-α-Act was 55°C and fluorescence captured at 82.5°C, while 58°C and 84°C were used for G3PDH. Product specificity was confirmed by melting curve analysis (55°C to 95°C ramp, 0.1°C per s), the identification of expected product T_m (85.7°C

for Sk-α-Act; 86.6°C for G3PDH), and by agarose gel electrophoresis of each amplicon (Figure 1). For each gene, mRNA transcript abundance was quantified by the inclusion of linear internal standards generated by PCR subcloning. Each sample was analyzed in duplicate within the same Smart Cycler run. Data was accepted when the standard curve had an $R^2 \geq 0.98$ and individual samples had a CV of less than 5%.

Cloning of Internal PCR Standards

Each target amplicon was subcloned into pGEM-T Easy vector (Promega) as described by the manufacturer's. Amplicons were generated from 1 μL of porcine muscle cDNA from mature skeletal muscle RNA in a 10 μL reaction, which contained 10X reaction buffer, magnesium chloride (2.5 m*M*), dNTPs (20 n*M*), gene specific primers (5 m*M* each), and Taq DNA polymerase (0.5 U; Promega). After forty cycles of amplification (95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 15 s) on a standard thermal cycler, PCR products were ligated into pGEM vector overnight at 4°C. Plasmid DNA was purified (Plasmid Midi Kit, Qiagen, Valencia, CA) and eluted in H_2O (pH 8.0). Insert identity was confirmed by sequence analysis on an ABI 377 sequencer (Applied Biosystems, Inc.) at the Iowa State University DNA Sequencing Facility with T7 (5'-AAT ACG ACT CAC TAT AG-3') primer. Sequences were compared with entries in GenBank by BLAST analysis to assure homology to known porcine sequences. Plasmid DNA was linearized by digestion with *Apa*I, quantified (A_{260}), and serially diluted to form a linear internal standard for inclusion in PCR reactions. Copy number for linear plasmid standards

was estimated with the following equation: 6 x 10^{23} (copies/mol) x plasmid concentration (g/μL) x MW^{-1} (g/mol).

Luciferase Expression Plasmid Construction

The generation of porcine genomic clones and characterization of the porcine Sk-α-Act promoter has been described (Reecy et al., 1998; Reecy et al., 1996). The chloramphenicol acetyltransferase (CAT) cDNA was removed from pPSKAFL-CAT (Reecy et al., 1996) by restriction endonuclease digestion with *EcoR*I and *Sal*I and replaced with a firefly luciferase (LUC) cDNA from pGL3-basic (Promega, Madison, WI) to produce pPSKA-LUC. The newly formed luciferase encoding plasmid contained 1929 bp of 5' flanking sequence, the first exon and 188 bp of the first intron of the porcine Sk-α-Act gene (Figure 3). Promoter deletion constructs were generated by endonuclease digestion of pPSKA-Luc with *BamH*I, *Xba*I, *Sma*I, or *Xho*I and *Hind*III similar to Reecy et al. (1998). Restriction fragments of expected length were gel purified (Qiagen QIAquick Gel Extraction Kit) and ligated, in frame, into pGL3-BASIC (Promega) 5' of the firefly luciferase reporter gene, to form pPSKA-Bam (nucleotides –550 to +243 of the porcine skeletal α-actin gene), pPSKA-Xba (-388 to +243), pPSKA-Sma (-142 to +243), and pPSKA-Xho (-84 to +243) (Figure 5). Plasmid DNA was purified (Qiagen Plasmid Midi Kit) and eluted in Tris-EDTA (pH 7.4) for storage at –20°C.

Transient Transfections

Transient transfection procedures were utilized to introduce luciferase constructs into primary cell cultures. Cells were transfected at 65 to 70% confluence with 2 μg of total purified plasmid DNA with Fugene 6 (3 μL; Roche Applied Science,

Indianapolis, IN) as described in the manufacturer's protocol in antibiotic-free GM. In all experiments, a firefly luciferase reporter construct (1 μg) was co-transfected with a Rous Sarcoma Virus (RSV) promoter driven β-galactosidase reporter plasmid (1 μg), RSV-βGAL (Reecy et al., 1997), to normalize for transfection efficiency. pGL3-Basic (Promega), which lacks a defined eukaryotic promoter, was transfected in parallel cultures to determine background luciferase expression. After 18 h of incubation, transfection media was removed and replaced with GM. Longer incubations in antibiotic-free media proved to be detrimental to primary cell survival (data not shown). Transfected cells were stimulated to differentiate and were treated with ractopamine (10 μ*M*) in serum-free media as described above.

Reporter gene assays

At times indicated, transfected cultures were rinsed twice with PBS and lysed by the addition of 220 μL of 1X reporter lysis buffer (Promega) for 10 min at room temperature. Cultures were scraped and lysates were transferred to a 1.5 mL centrifuge tube. To ensure complete lysis, three cycles of rapid freeze (liquid N_2) and thaw (37°C H_2O bath) were performed on collected samples. Cell debris was removed by centrifugation at 20,000x *g* for 2 min at 25°C. Twenty microliters of lysate was mixed with 100 μL of luciferase substrate (20 m*M* Tris, 4 m*M* $MgSO_4$, 0.1 m*M* EDTA, 30 m*M* DTT, 0.5 m*M* ATP, 0.25 m*M* Co-enzyme A, 0.5 m*M* D-luciferin pH 8.0) in a standard luminometer (TD-20/20; Turner Designs, Sunnyvale, CA) and chemiluminescence (expressed as relative light units, RLU) was measured for 15 s. β-Galactosidase activity was determined spectrophotometrically (420 nm) as

described (Rosenthal, 1987). Briefly, 50 µL of cellular lysate was incubated at 37°C for 6 to 18 h in 450 µL of Z buffer (2.70 µL/mL β-mercaptoethanol, 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgSO_4$ pH 7.0) supplemented with 100 µL of 2-Nitrophenyl β-D-galactopyranoside (ONPG; Sigma). To determine total culture protein levels, 10 µL of cell lysate was loaded onto a 96-well microtitre plate and protein concentration was determined by the Bradford assay (Bio-Rad Corp, Hercules, CA) with a linear BSA standard as per the manufacturer's recommendations with a SPECTRAmax $340PC^{384}$ plate reader and Softmax Pro 4.0 software (Molecular Devices, Sunny Vale, CA). Luciferase activity was normalized to β-galactosidase activity and expressed as RLU per ng of total protein.

Statistical Analysis

Data was analyzed by one- or two-way analysis of variance (ANOVA) with JMP software (Release 5.0, 2001; SAS Institute Inc., Cary, NC). For luciferase expression assays, multiple experiments were pooled and a mixed model was applied to the composite data. The main effects of treatment, time (or construct), and respective interactions were determined, while the effects of experiment and experiment interactions were treated as random. When main effects were significant, least squared means (LSM) differences were determined by Tukey's HSD procedure, *post hoc*. Least squared means and the standard error of the mean are reported. Comparisons were significantly different at the level of $P < 0.05$.

Results and Discussion

Quantitative PCR

A two-step real-time quantitative RT-PCR approach was used to determine the relative mRNA abundance of Sk-α-Act in both ractopamine-stimulated and untreated porcine myotubes. This technique was optimized to minimize the amplification of non-specific amplicons (Figure 1). After 72 h in SFDM supplemented with ractopamine, SK-α-Act mRNA abundance increased more than six-fold relative to untreated control cultures (Table 1). Untreated myotube cultures contained approximately 12,800 copies of Sk-α-Act mRNA transcripts per picogram of total RNA, whereas ractopamine treated cultures contained 85,200 copies (Table 1).

Although the magnitude of response was greater in this study, our findings are in agreement with *in vivo* studies in which ractopamine increased SK-α-Act mRNA levels (Grant et al., 1993; Helferich et al., 1990; Liu et al., 1994). Similarly, skeletal α-actin mRNA abundance increased *in vitro* when ventricular myocytes were maintained in serum-free media supplemented with the β-adrenergic agonist isoproterenol (Bishopric et al., 1992b). In this study, the effect of β-adrenergic agonists on Sk-α-Act expression has been recapitulated in porcine myotubes maintained in SFDM.

Average Protein Concentrations in Porcine Myotubes Treated with Ractopamine

Protein synthetic rate has been documented to peak at 24 h in zinterol treated L8 muscle cells (McElligott et al., 1989), between 24 and 48 h in clenbuterol treated

L6 cultures (McMillan et al., 1992), and after 24 h in ractopamine treated ELC5 myotubes (Anderson et al., 1990). In this study, total protein concentrations were measured *post hoc* to determine if ractopamine increased protein synthesis during a time course that spanned 72 h in treatment media.

The average protein content in harvested porcine myotubes was unchanged after treatment with ractopamine at 24 and 48 h, but tended to be higher after 72 h ($P = 0.07$; Figure 2). However, culture protein concentrations decrease in a time-dependent manner. It should be noted that the serum-free treatment media described in this study contained the synthetic glucocorticoid dexamethasone at a final concentration of 2.5 μM. This level was previously reported to be optimal to increase β-adrenergic receptor populations in bovine myotubes as well as the apparent synthetic rate of myosin heavy chain in response to 1 μM isoproterenol (Bridge et al., 1998). Our results are similar to those obtained by Desler et al. (1996), in which total cellular protein deceased a dose-dependent manner in C2C12 myotubes treated with dexamethasone in serum-free. Though we did not measure the apparent protein synthesis or degradation rates in this study, we believe that our results suggest that porcine myotubes may be more sensitive to this level of dexamethasone (2.5 μM) than bovine myotubes and, even in the presence of ractopamine, protein degradation may exceed synthetic rates, which explains the trends observed (Figure 2). However, dexamethasone does not adversely affect Sk-α-Act expression at the mRNA level (Koukouritaki et al., 1997, and this study), thus, these results do not directly affect the conclusions that may be drawn herein as our

objective was to measure transcriptional responsiveness of the Sk-α-Act promoter to ractopamine.

Skeletal-α-Actin Promoter Responsiveness to Ractopamine

In order to establish if ractopamine directly regulates the transcription of the porcine skeletal α-actin gene, luciferase reporter gene assays were performed. The porcine Sk-α-Act promoter, which contained ~1.9 kilobases (kb) of 5' DNA sequence (relative to the transcriptional start site), the first exon (55 bp), and 188 bp of the first intron, was cloned 5' of luciferase cDNA to produce a 6.8 kb chimeric vector (Figure 3). As previously reported (Reecy et al., 1998), this region of genomic DNA regulates the expression of porcine Sk-α-Act in a developmental and muscle specific manner. Although fibroblast contamination was never a significant problem in our primary cell culture model (data not shown), our luciferase promoter constructs were designed to retain muscle specific expression, which was conferred by an enhancer in the first intronic region (Reecy et al., 1998). Initial transfection experiments in untreated C2C12 myotubes, C2C12 myoblasts, and 10T ½ fibroblasts confirmed that all constructs used in this study behaved in a developmental and tissue specific manner (data not shown).

In order to establish a time course for subsequent experiments, the full-length pPSKA-LUC plasmid was transfected as described into porcine myoblasts. Treated myotubes were harvested in 24-h increments and luciferase activity was evaluated. The cloned Sk-α-Act promoter did not respond to ractopamine after as many as 72 h in SFDM (Figure 4). However, basal promoter activity increased over time ($P <$

0.001). As depicted in Figure 5, experiments conducted with luciferase expression constructs driven by truncations of the full-length promoter further failed to respond to ractopamine after 72 h. From these experiments, we were unable to determine if a ractopamine responsive DNA element exists in the 5' flanking region (-1929 to +243 bp) of the porcine Sk-α-Act encoding gene.

The constructs used in this study behaved similarly to previous reports that utilized the Sk-α-Act promoter; transcriptional activity decreased as the regions distal to the transcriptional start site were deleted (Bishopric & Kedes, 1991; Reecy et al., 1998). Interestingly, Reecy et al. (1996) identified an enhancer region within the distal region of the porcine Sk-α-Act promoter (-1929 to –550 bp) that was required to confer a high level of transcriptional responsiveness in C2C12 myotubes. However, this region does not appear to have an enhancer-like function in porcine myotubes as the luciferase activity was not different when the full-length construct (-1929 to +243 bp) was compared to the both the pPSKA-Bam (–550 to +243) and pPSKA-Xba (-388 to +243) constructs regardless of ractopamine stimulation (data not shown). Nonetheless, transcriptional responsiveness was diminished, relative to the full-length promoter, in the absence of the serum-response elements (SRE) between –388 and –142 bp ($P < 0.01$) and in absence of the SRE in the proximal DNA region (-142 bp to –84; $P < 0.001$) of the Sk-α-Act promoter, which is consistent with previous reports (Bishopric & Kedes, 1991; Reecy et al., 1998).

The expression, or in some species, re-expression, of Sk-α-Act is associated with cardiac hypertrophy and hypertension (Schwartz et al., 1992; Schwartz et al., 1993) and, therefore, the adrenergic regulation of this gene has been most closely

examined in the context of cardiac, rather than skeletal, muscle hypertrophy. Interestingly, norepinephrine increased transcriptional activity of an enhancer-containing human Sk-α-Act promoter (-2000 to +187 bp) by 2.4-fold in high-density cardiac myocytes via activation of the β-adrenergic receptors (Bishopric & Kedes, 1991). In that study, a DNA region between –2000 and –36 bp (relative to the transcriptional start site) conferred norepinephrine responsiveness. A DNA region located between -153 and -36 bp, which contains a binding site (-90 bp) for the transcription factor activator protein-1 (AP-1), was demonstrated to be necessary for isoproterenol-induced transactivation of the human Sk-α-Act promoter by a c-fos/c-jun heterodimer in cardiac myocytes (Bishopric et al., 1992a). This region is conserved between the human and porcine Sk-α-Act genes (Reecy et al., 1996). It is, therefore, unclear why both ractopamine and isoproterenol (data not shown) failed to induce the porcine Sk-α-Act promoter in a similar manner in porcine myotubes. Together with the increased pretranslational Sk-α-Act expression in our system, it is likely that DNA regulatory elements not included in the chimeric expression vectors described herein confer transcriptional responsiveness to ractopamine, if mRNA abundance is indeed a function of increased Sk-α-Act transcription.

Alternatively, the increased Sk-α-Act mRNA abundance observed in response to ractopamine may result from increased mRNA stability. While this has not been previous postulated for ractopamine, other β-adrenergic agonists have been shown to positively and negatively regulate gene expression via post-transcriptional

mechanisms. Beta-actin mRNA abundance transiently increased independent of an increased nuclear transcription rate in the parotid glands of isoprenaline treated mice (Roberts et al., 1991). Conversely, a heteronuclear protein, hnRNP A1, selectively reduces the half-life of β_1-adrenergic receptor mRNA transcripts in rat C6 glioma cells treated with isoproterenol (Kirigiti et al., 2001). In this case, hnRNP A1 binds to the 3' untranslated region (UTR) of the β_1-adrenergic receptor transcript to confer decreased mRNA stability. It is conceivable that ractopamine may selectively increase or decrease mRNA stability in a similar manner. With respect to Sk-α-Act and the apparent ractopamine-mediated increase in pre-translational expression, the 3' UTR has not been studied. As Sk-α-Act mRNA abundance increases independent of a global increase in mRNA transcription (Grant et al., 1993; Helferich et al., 1990), further investigation into the post-transcriptional control of gene expression appears to be warranted.

Implications

While other studies have provided evidence that ractopamine regulates the expression of Sk-α-Act and possibly myosin heavy chain isoforms (Depreux et al., 2002; Shappell et al., 2000), the molecular mechanisms underlying these effects are poorly understood. This is the first study to look specifically at the effects of ractopamine on the transcriptional regulation of a contractile protein in myotube cultures. Our model is sufficient to evaluate the anabolic effects of ractopamine in porcine skeletal muscle. Unfortunately, we were unable to identify a cis-acting DNA

element within the cloned Sk-α-Act that conferred transcriptional responsiveness to ractopamine in porcine skeletal muscle. Further evaluation of the distal regulatory elements in the Sk-α-Act locus and/or the 3' UTR of the gene may provide mechanistic detail as to how ractopamine promotes the accumulation of Sk-α-Act mRNA in porcine skeletal muscle.

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Figures and Tables

Figure 1. Agarose gel electrophoresis of amplicons generated from porcine myotubes by real-time quantitative PCR (10 μL of PCR product per lane). Lane 1, 1-kb ladder; lanes 2 and 3, Sk-α-Act and G3PDH amplified from ractopamine treated myotubes; lanes 4 and 5, Sk-α-Act and G3PDH amplified from control cultures. Amplification of G3PDH served as a normalization factor for comparing relative expression of SK-α-Act. Sizes of PCR products are indicated in basepairs (bp).

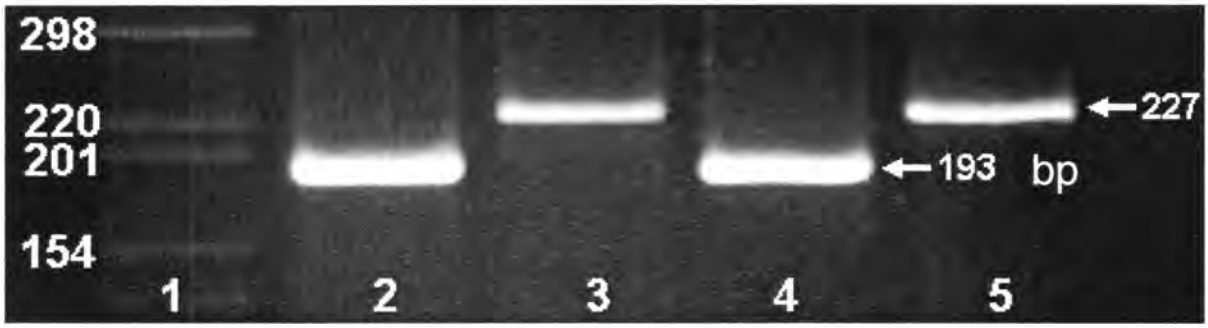


Table 1. Detection of skeletal-α-actin mRNA in porcine myotubes as determined by real-time quantitative PCR. Transcript copy number was estimated from linear internal standards and is expressed per picogram (pg) of total RNA. Values represent the average of duplicate measures on the same pool of cDNA.

	Estimated Copies per pg Total RNA	
Treatment [a]	Skeletal α-Actin [b]	Fold Change [c]
Control	12800	1.00
Ractopamine	85200	6.65

[a] Applied to differentiated myotubes for 72 hours in serum free media.
[b] Derived from linear internal standards and normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression.
[c] Expressed relative to time matched control.

Figure 2. Average protein concentrations in porcine myotubes. Myotubes were harvested after 0, 24, 48, and 72 hours in treatment media. Concentration is expressed as nanograms (ng) per culture. One-way ANOVA was performed within time point. † Treatment tended to be significantly different, ($P = 0.07$).

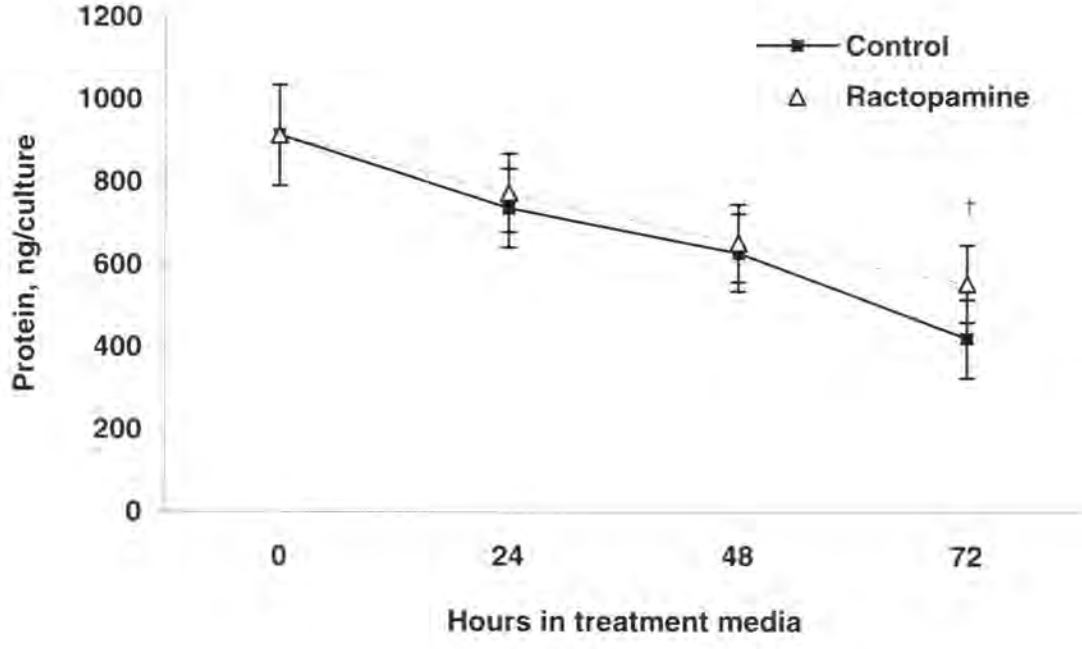


Figure 3. Luciferase expression vector, which contained 2.1 kb of the porcine skeletal α-actin promoter. Conserved cis-acting DNA elements and transcription factor binding motifs are depicted (legend). Sites specific for restriction endonucleases used to generate truncated promoter constructs: B, *BamH*I; Xb, *Xba*I; S, *Sma*I; Xh, *Xho*I; H, *Hind*III.

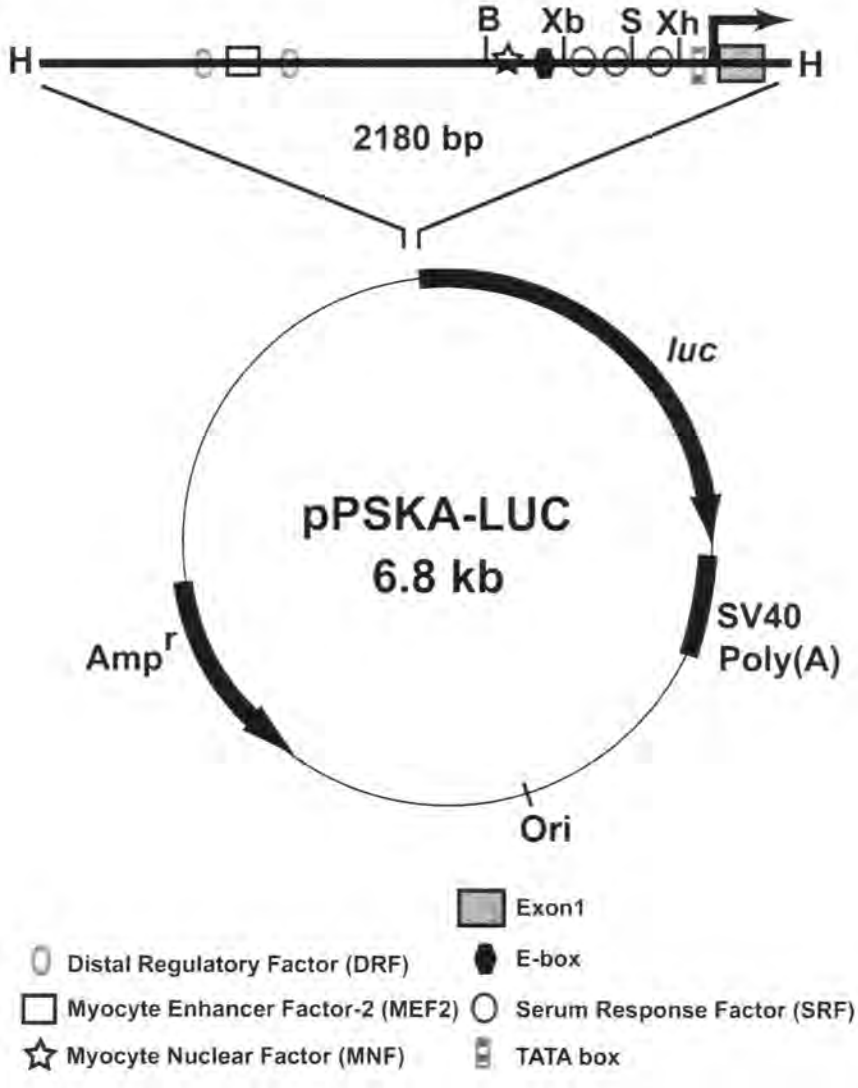


Figure 4. Relative luciferase activity from porcine myotubes transfected with full-length porcine skeletal-α-actin promoter construct (pPSKA-LUC). Transfected myotubes were harvested after 0, 24, 48, and 72 hours in treatment media. Activity is expressed as relative light units (RLU) per nanogram of lysate protein. Effects: treatment ($P > 0.10$), time ($P < 0.001$), time x treatment ($P > 0.10$).

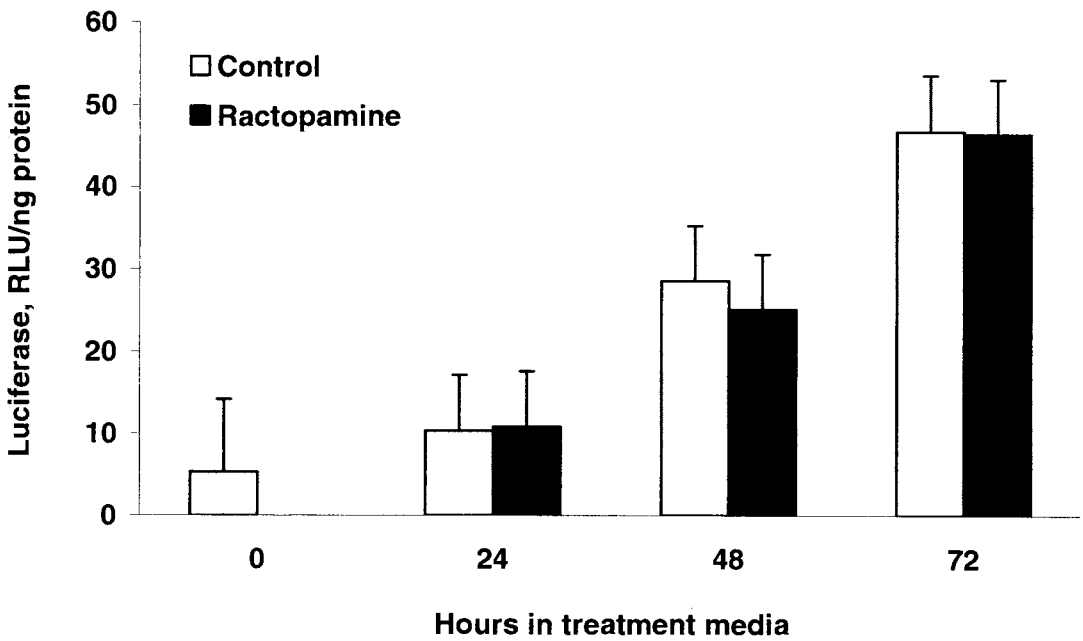
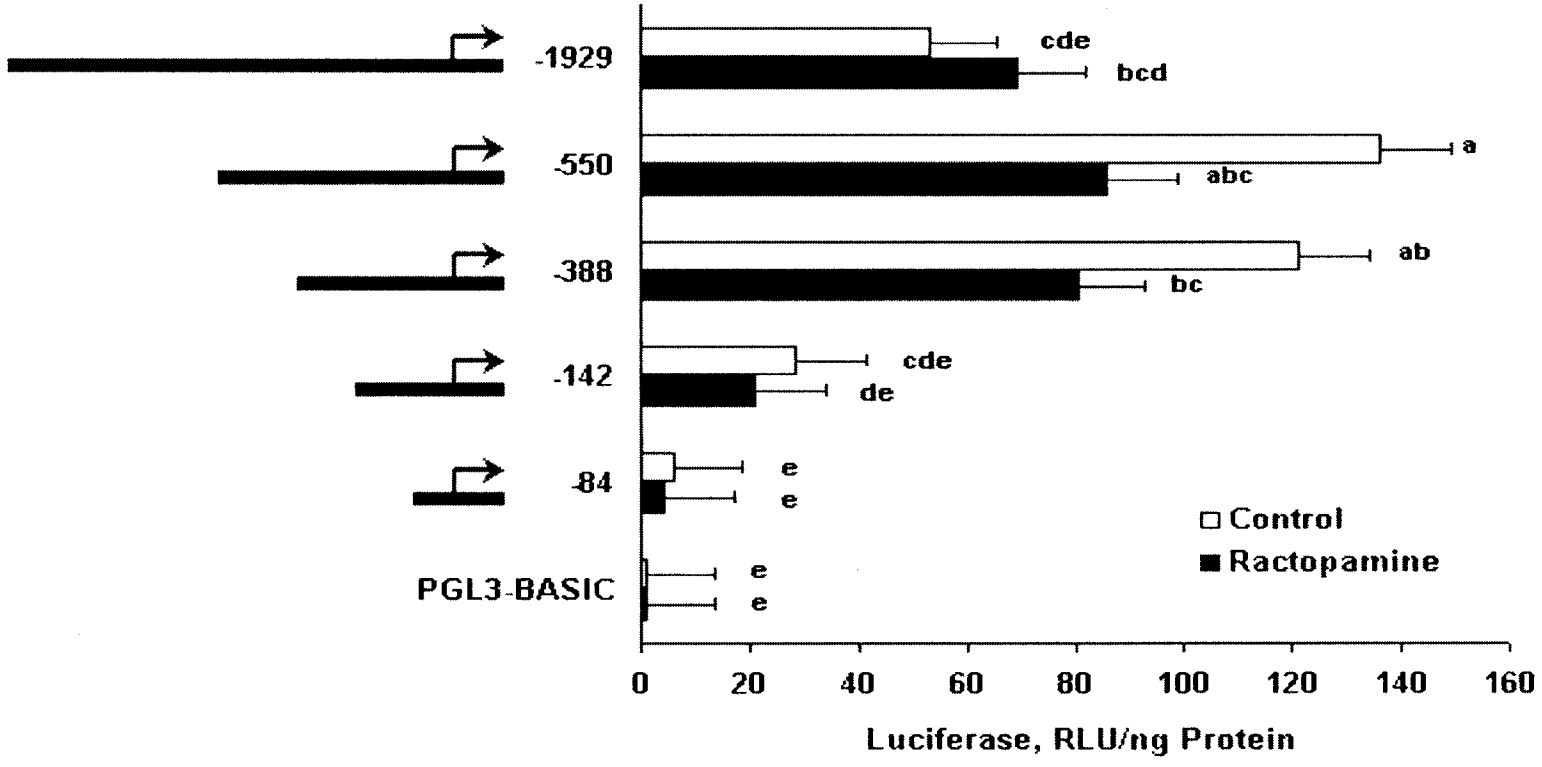


Figure 5. Luciferase activity from porcine myotubes transfected with skeletal-α-actin promoter constructs. Myotubes were harvested after 72 hours in treatment media. Activity is expressed as relative light units (RLU) per nanogram (ng) of lysate protein. Effects: treatment ($P < 0.05$), construct ($P < 0.001$), construct x treatment ($P < 0.05$). Means with different letters are different ($P < 0.05$).



CHAPTER 3. DIFFERENTIAL GENE EXPRESSION IN LONGISSIMUS DORSI MUSCLE OF PIGS FED RACTOPAMINE

A paper prepared for submission to the Journal of Animal Science

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Abstract

Ractopamine, commercially available as Paylean, promotes hypertrophy and alters myosin heavy chain (MHC) isoform content in porcine skeletal muscle, presumably through molecular mechanisms that involve the activation of β-adrenergic receptors and associated intracellular signal transduction cascades. However, sufficient mechanistic detail as to how muscle hypertrophy and fiber type plasticity is regulated by ractopamine has proven to be elusive at the molecular level. We used the suppression subtractive hybridization (SSH) procedure to examine pre-translational gene expression after ractopamine administration in porcine skeletal muscle. Longissimus dorsi muscle samples were collected after feeding two levels of ractopamine (Paylean at 0 or 60 ppm) to maternal line pigs for 3 d and polyadenylated (poly A^+) mRNA was used to generate cDNA libraries for each treatment. Using the control (0 ppm) cDNA library as the driver and the

ractopamine (60 ppm) cDNA library as the tester, a forward-subtracted cDNA library was generated by SSH. After amplification by PCR, the resultant amplicons were subcloned and twenty-four of 192 clones were selected for sequence analysis. Differentially expressed genes were putatively identified by sequence homology (>85%) to GenBank entries. Nine genes (calmodulin-1, phosphatase inhibitor 2, homer homolog 1, mortalin-2, DnaJ (Hsp40) homolog, Mago-nashi homolog, heterogeneous nuclear ribonucleoprotein, synthrophin, and synemin) and one expressed sequence tag (EST) were identified as being differentially expressed in longissimus dorsi muscle at the pre-translational level after ractopamine stimulation. Northern blot analysis performed on the original RNA samples confirmed that calmodulin-1 mRNA abundance increases approximately 2-fold ($P < 0.05$) after 3 d of ractopamine treatment. This is the first study in which the expression of a calcium-modulated protein has been implicated in the phenotypic adaptations of skeletal muscle to ractopamine in the pig. Further elucidation of the transcriptional control of calmodulin-1 by ractopamine, as well as the post-translational role of this protein, should provide more detail as to how ractopamine alters skeletal muscle phenotype and enhances lean body composition.

Keywords: ractopamine, gene expression, porcine, skeletal muscle

Introduction

In the pig, the β-adrenergic agonist ractopamine stimulates muscle hypertrophy (Beermann, 2002; Kim & Sainz, 1992) and increases lean carcass

composition (Watkins et al., 1990). Increases in protein synthetic rate (Adeola et al., 1992; Bergen et al., 1989), changes in myosin heavy chain (MHC) isoform content (Depreux et al., 2002), and increases in the pre-translational expression of skeletal muscle specific genes (Grant et al., 1993; Helferich et al., 1990) have been observed in porcine skeletal muscle after feeding ractopamine. These effects appear to be the result of changes in many biological processes, which may include enzymatic activity, substrate utilization, protein synthetic rate and/or translation efficiency, and gene transcription. However, sufficient detail as to how ractopamine may directly alter these physiologically relevant processes has remained largely elusive at the molecular level.

Modified gene expression accompanies phenotypic changes when skeletal muscle undergoes both hypertrophy (Carson, 1997; Goldspink, 2002) and atrophy (Bey et al., 2003; Wittwer et al., 2002). While not a prerequisite, sustained increases in the steady-state mRNA levels of proteins involved in ion transport, biochemical pathways, cellular integrity, and signal transduction pathways tend to indicate cellular adaptations to stimuli that promote hypertrophy as well as fiber type plasticity. The identification of genes that are pre-translationally regulated by trophic, hormonal, or mechanical stimuli provides a framework from which to develop innovative hypothesis-driven research, as this approach often implicates novel signal transduction pathways and molecular events that are not fully appreciated. The aim of this study was to identify genes in porcine skeletal muscle that are potentially regulated by ractopamine in order to further illustrate the molecular mechanisms involved in both skeletal muscle hypertrophy and fiber type plasticity.

Materials and methods

Animals and Tissue Collection

Longissimus dorsi muscle samples were harvested from a subset of animals used in a larger study previously reported (Depreux et al., 2002). Four animals per treatment (0 and 60 ppm Paylean) were slaughtered after 3 d. Longissimus muscle samples were removed and snap frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

RNA Isolation and Poly(A^+) mRNA Synthesis

Total RNA was isolated with the RNeasy Midi kit (Qiagen, Valencia, CA) as described in the manufacturer's protocol. Briefly, longissimus dorsi samples (0.25 g) were individually homogenized in 3.0 mL of Buffer RLT (Qiagen) with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Homogenates were digested with Proteinase K (20 mg/mL; Qiagen) at 55°C for 20 min. After removal of tissue debris by centrifugation, cleared lysates were loaded onto RNeay Midi column and rinsed by the addition of 4.0 mL of Buffer RW1 followed by two rinses with Buffer RPE (2.5 mL) as described in the kit's protocol. Total RNA was eluted in two volumes of Rnase-free water (500 μL total) and quantified (A_{260}). Equal amounts of total RNA (0.25 mg) from each animal were pooled by treatment (1.0 mg final) and poly A^+ RNA was purified with the Oligotex mRNA kit (Qiagen) as described in the manufacturer's protocol. Briefly, total RNA pools were incubated in Buffer OBB (500 μL) and Oligotex Suspension (55 μL) for 3 min at 70°C. The Oligotex:mRNA complexes were isolated by centrifugation, resuspended in 400 μL Buffer OW2, and

applied to small spin columns. After rinsing (400 μL of Buffer OW2), poly A^+ RNA was eluted in 100 μL of Buffer OEB, which had been pre-warmed to 70° C. Purified poly A^+ RNA was quantified (A_{260}) and stored at -80°C.

cDNA Library Synthesis

For each treatment pool (0 ppm and 60 ppm Paylean), a cDNA library was generated from poly A^+ RNA with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and second-strand synthesis procedures outlined in the Clonetech PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA). Briefly, 0.75 mg of poly A^+ RNA was denatured at 65°C for 10 min and first-strand synthesis was initiated by the addition of bulk first-strand reaction mix (5 μL; Amersham), 1,4-Dithio-DL-threitol (DTT; 1 μL), cDNA synthesis primer (10 μ*M*; Clonetech), and RNAse-free water to a final volume of 20 μL. The reaction mixture was incubated at 37°C for 1 h. Second-strand synthesis was initiated by the addition of 5X second-strand buffer (16.0 μL), dNTP mix (10 m*M*), 20X second-strand enzyme cocktail (4.0 μL; Clonetech), and RNAse-free water to a final volume of 80 μL. The reaction was incubated at 16°C for 2 h and terminated by the addition of 4 μL of 20X EDTA/glcogen mix (0.2 *M* EDTA; 1 mg/mL glycogen). Generated cDNAs were isolated by phenol:chloroform extraction and ethanol precipitation as directed by the manufacturer's protocol (Clonetech).

Suppression Subtractive Hybridization

A polymerase chain reaction (PCR)-based cDNA suppression subtractive hybridization (SSH) was performed on the two generated cDNA libraries in order to

identify differentially expressed mRNAs in longissimus muscle of Paylean fed pigs. The Clonetech PCR-Select cDNA Subtraction Kit (Clontech) was used as described in the manufacturer's protocol. The cDNA library generated from pigs fed 60 ppm Paylean was designated "tester" and the cDNA library from pigs fed 0 ppm Paylean was designated "driver." Both cDNA libraries were digested with the restriction endonuclease *Rsa*I (15 U) in 10X restriction digest buffer (New England Biolabs, Beverly, MA) at 37°C for 1.5 h. After phenol:chloroform extraction, tester cDNA populations were divided into two microcentrifuge tubes and ligated with either adapter 1 (10 μ*M*) or adapter 2R (10 μ*M*) in 5X ligation buffer with T4 DNA ligase (400 U). Prior to overnight ligation at 16°C, 2 μL of each ligation mixture was reserved and diluted into 1 ml sterilized water to serve as an unsubtracted control in later procedures. No adapter sequence was ligated to the driver (0 ppm Paylean) cDNA library. After the confirmation of successful adapter ligation by PCR, the first subtractive hybridization was performed by the addition of *Rsa*I-digested driver cDNA (1.5 μL) and 4X hybridization buffer (1.0 μL; Clonetech) to microcentrifuge tubes that contained either 1.5 μL of adapter 1-ligated or 1.5 μL of adaptor 2R-ligated tester cDNA. The mixture was denatured for 1.5 min at 95°C and incubated at 68°C for 8 h. A second hybridization was performed by simultaneously combining both samples and 1 μL of freshly denatured driver cDNA. Hybridization was allowed to proceed overnight at 68°C. Subtracted samples were diluted by the addition of 200 μL dilution buffer (20 m*M* HEPES pH 8.3, 50 m*M* NaCl, and 0.2 m*M* EDTA), heated for 10 min at 68°C, and stored at -20°C.

PCR amplification of Subtracted Products

Two PCR amplifications were performed on the subtracted and unsubtracted products. For the primary PCR amplification, 1 μL of subtracted or unsubtracted cDNAs was combined with 10X PCR reaction buffer (2.5 μL), dNTP mix (10 m*M*), PCR primer 1 (Clonetech), 50X Advantage cDNA Polymerase Mix (0.5 μL; Clonetech), and sterile water to a final volume of 25 μL. Polymerase chain reaction conditions were: 75°C for 5 min followed by 28 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 1.5 min. Amplified PCR products were diluted ten-fold in sterile water and 1 μL of diluted primary PCR products were combined with 10X PCR reaction buffer (2.5 μL), dNTP mix (10 m*M*), nested PCR primer 1 (10 μ*M*; Clonetech), nested PCR primer 2R (10 μ*M*; Clonetech), 50X Advantage cDNA Polymerase Mix (0.5 μL; Clonetech). Polymerase chain reaction conditions for secondary amplification were: 94°C for 30 s followed by 25 cycles of 68°C for 30 s and 72°C for 1.5 min. Primary and secondary PCR products were analyzed by gel electrophoresis (Figure 1).

Cloning of Amplified Subtraction Products for Sequence Analysis

Freshly amplified secondary PCR products were subcloned into pGEM-T Easy vector overnight at 4°C as described by the pGEM-T Easy Vector System protocol (Promega, Madison, WI). Ligation reactions were transformed into JM109 High Efficiency Competent Cells (Promega) and 100 μL of each transformation was plated on LB/ampicillin/isopropyl-beta-D-thiogalactopyranoside (IPTG)/ 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) plates, which were incubated at 37°C overnight as recommended. Blue/white color screening was used to select

192 colonies for further analysis. Plasmid DNA was extracted and purified in a 96-well format as previously described by Marra et al. (1997). Briefly, 1.2 mL of Terrific Broth II (TB; Bio 101, Inc., Carlsbad, CA) was supplemented with ampicillin (50 mg/mL) and inoculated with a single colony. Cultures were grown in 2-mL 96-well blocks for 18 h at 37°C with agitation at 300 rpm in a shaking incubator. After growth, glycerol stocks were prepared by combining 50 μL of culture with 50 μL of 30% glycerol in TB in 96-well microplates. Microplates were sealed and glycerol stocks were maintained at –80°C. Bacterial cultures were pelleted by centrifugation at 1730x *g* for 15 min at 4°C. Pellets were resuspended in 100 μL of GET/RNase buffer (90 mg/mL dextrose, 25 m*M* Tris-HCl pH 8.0, 10 m*M* EDTA pH 8.0, 20 mg/mL RNase A (Sigma Chemical Co., St. Louis, MO)) and lysed by the addition of 200 μl of cell lysis solution (0.2 *N* NaOH/ 1% SDS) followed by agitation for 5 min at room temperature. The lysis reaction was terminated by the addition of 200 μL of 3 *M* potassium acetate (KAc; pH 4.8) followed by incubation at 4°C for 30 min. Cellular debris was pelleted by centrifugation (3000x *g* for 20 min at 4°C) and the cleared lysate was applied to UniFilter 96-well filter plates (Whatman, Inc., Clifton, NJ) in two aliquots of 200 μL. The UniFilter plates were then secured above UniPlate 96-well collection plates (Whatman) that contained 300 μL of isopropanol. The plates were centrifuged at 3100x *g* for 80 min at 0°C to purify and precipitate plasmid DNA. Precipitated DNA was resuspended in 50 μL of sterile water and stored at –20°C.

Sequence analysis

Prior to sequence analysis, 5 μL of purified plasmid DNA was digested overnight with the restriction endonuclease *EcoR*I at 37°C in a reaction that contained 10X reaction buffer (Promega), acetylated bovine serum albumin (BSA; 0.1 mg/mL), *EcoR*I (3 U; Promega), and sterilized water to a final volume of 10 μL. Digestion products were size separated and visualized by gel electrophoresis (1% agarose). As the cloning vector used in this study (pGEM) contains two *EcoR*I restriction sites that flank the multiple cloning site, this step allowed for the rapid identification and selection of multiple cDNA inserts of various sizes for sequence analysis. Twenty-four unique clones were selected and 15 μL of purified plasmid DNA was sequenced on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA) at the Iowa State University DNA Sequencing Facility with SP6 (5' GAT-TTA-GGT-GAC-ACT-ATA-G 3') primer. Clone identity was predicted by BLAST analysis and putative identification was assigned to clones that had ≥ 85% homology to a known gene, homologue, or expressed sequence tag (EST). Cloned sequences were also compared to porcine-specific (Sus scrofa) EST clones deposited in GenBank. Tentative consensus (TC) sequence identity was assigned after comparison with the Institutive for Genomic Research (TIGR) Porcine Gene Index (SsGI) database.

Confirmation of Differential Expression by Northern Blot Analysis

The differential expression of genes recognized to be involved in signal transduction networks was confirmed by Northern blot analysis. Total RNA was collected from 0.5 g of the original longissimus dorsi samples using TRIzol reagent

(Invitrogen Corporation, Carlsbad, CA) as directed by the manufacturer's supplied protocol. Total RNA (20 μg) from each individual pig and from the two treatment pools identical to that used for SSH was size separated by electrophoresis through a 1% agarose denaturing formaldehyde gel (Ambion, Inc., Austin, TX). RNA was transferred to a positively charged nylon membrane (BrightStar-Plus; Ambion) and UV cross-linked (120,000 μJoules; Stratalinker 2400, Stratagene, La Jolla, CA). Riboprobes were generated with the Strip-Ez kit (Ambion). The differential expression of calmodulin-1 was confirmed with an antisense complementary RNA (cRNA) probe synthesized from a sequenced clone, designated pGEM-CaM1, which was 87% homologus to the 3' untranslated region of the human calmodulin-1 mRNA sequence (GenBank Accession No. U16850). Briefly, 1 μg of pGEM-CaM1 was linearized by restriction digest with *Dra*III (Promega) and gel purified (Qiagen Gel Extraction Kit). Linearized plasmid DNA (50 ng) was incubated at 40°C for 2 h in a reaction that contained 10X transcription buffer (Ambion), ATP (5 m*M*), CTP (5 m*M*), GTP (5 m*M*), 800 Ci/mmol [α-^{32}P] UTP (3.125 μ*M*), T7 RNA polymerase mix (10 U; Ambion), and Rnase-free water to a final volume of 20 μL. After digestion with Rnase-free DNase I (10 U; 37°C for 15 min) and column purification (Bio-spin column; Bio-Rad Corp, Hercules, CA), the synthesized cRNA probe was hybridized to the Northern blot overnight at 68°C with agitation in 30 mL of Ultra-Hyb buffer (Ambion). The membrane was washed once in Low Stringency Wash Solution #1 (Ambion Northern Max Kit) for 5 min with agitation at room temperature and two consecutive times with High Stringency Was Solution #2 for 15 min at 68°C. The hybridization signals were obtained using by exposing the hybridized blot to a

PhosphorImager screen at room temperature for 24 h and scanning the screen with a Molecular Dynamics Storm 860 PhosphorImager System (Amersham). Hybridization signals were quantified with version 1.2 of ImageQuant for Macintosh (Amersham). Values were corrected against the β-actin signal obtained from cRNA probes generated with the pTRI-β-actin-Mouse template DNA supplied in the Strip-EZ kit as recommended by the manufacturer. Riboprobes were synthesized for phosphatase inhibitor 2 and homer homolog 1b in a similar manner, but the resultant hybridization signal was below detection threshold by Northern blot analysis.

Statistical Analysis

Densitometry data was analyzed by one-way analysis of variance (ANOVA) with JMP software (Release 5.0, 2001; SAS Institute Inc., Cary, NC). Least squares means and the standard error of the mean are reported. Comparisons were significantly different at the level of $P < 0.05$.

Results and Discussion

While phenotypic changes (muscle hypertrophy and fiber type plasticity) in skeletal muscle are well documented (Beermann, 2002; Kim & Sainz, 1992), little is known about the global changes in gene expression that occur after the administration of β-adrenergic agonists to livestock species. In the post-genomic era, the use of cDNA microarray analysis to facilitate gene expression profiling and discovery has provided insight into the genetic regulation of muscle adaptation in animal models of resistance training (Carson et al., 2002), muscle wasting (Wittwer

et al., 2002), muscular dystrophy (Haslett & Kunkel, 2002), and aging (Weindruch et al., 2001). Unfortunately, high-density cDNA microarrays have been slow to reach the laboratories of animal scientists and, only recently, has a porcine skeletal muscle cDNA microarray been developed and publicly reported (Bai et al., 2003; Yao et al., 2002). This study used an alternative method, suppression subtractive hybridization (SSH), to identify differentially regulated genes in the longissimus dorsi muscle of ractopamine treated pigs after 3 d of treatment. This technique allows for the selective enrichment of lowly expressed genes from a normalized cDNA library (Diatchenko et al., 1996). We were able to putatively identify nine previously unreported genes and one EST, which may be differentially expressed in skeletal muscle after stimulation with ractopamine.

In this study, SSH was performed on cDNA libraries generated from a subset of muscle samples collected for a larger study, which investigated myosin heavy chain (MHC) isoform abundance in skeletal muscle after feeding three levels of ractopamine (0, 20, or 60 ppm Paylean) to pigs for 3, 7, 14, 28, or 42 d (Depreux et al., 2002). We chose to use the longissimus dorsi muscle samples collected after 3 d from two treatment doses (0 and 60 ppm). Previously, pigs fed 60-ppm ractopamine grew faster (average daily gain) and had a relatively higher abundance of 2A MHC fibers in longissimus dorsi and semitendinosus muscles when compared to contemporary controls after 3 d (Depreux et al., 2002). Additionally, previous reports have indicated that the longissimus muscle is susceptible to hypertrophy, and increases in mass in response to ractopamine (Adeola et al., 1990; Stites et al., 1991; Yen et al., 1991). We hypothesized that differentially expressed genes

identified in porcine longissimus muscle after 3 d of ractopamine administration would be involved in the stimulation and/or the maintenance of muscle growth and fiber type plasticity.

We used poly A^+ mRNA to generate cDNA libraries to represent each treatment pool. After hybridization and PCR, the transcript size of the differentially expressed (forward-subtracted) cDNAs was analyzed by gel electrophoresis (Figure 1). Amplicon size ranged from approximately 200 to 1500 base pairs (bp), with three abundant amplicons, relative to the unsubtracted cDNA pool, visualized between 396 and 750 bp. Amplicons were subcloned, sequenced, and assigned putative gene identification after BLAST analysis.

Of the 24 clones sequenced, we were able to identify 22 by sequence comparison; poor sequencing reaction results prohibited the identification of two clones. Nine genes and one EST were identified as being potentially differentially expressed by SSH (Table 1). With the exception of one clone with low homology (82%) to synemin, isoform B, the sequenced clones were ≥ 85% homologus to known sequences deposited in GenBank. We observed several instances of redundancy between cDNA clones; in total, 12 of the 22 clones compared by sequence analysis shared homology with the same GenBank entry for four different genes (calmodulin-1 (3), phosphatase inhibitor 2 (4), DnaJ (Hsp40) homolog (3), and syntrophin (2)). The nine genes and one EST were categorized into functional clusters (Table 1) before further analysis. Sequenced cDNA clones were also compared with porcine EST sequences deposited into GenBank and were assigned a TC identity (Table 2). The putatively identified genes encode two structural

proteins, two chaperon proteins, two mRNA processing proteins, and three proteins that have been implicated in signal transduction pathways. We chose to confirm the differential expression of the one gene involved in the maintenance of calcium homeostasis and signal transduction pathways, calmodulin-1 (CALM1), in order to further elucidate the molecular mechanisms by which ractopamine affects muscle phenotype.

Northern blot analysis confirmed the differential expression of CALM1 in ractopamine treated skeletal muscle after 3 d (Figure 2A). After normalization to β-actin steady-state mRNA abundance, CALM1 mRNA levels increased approximately 2-fold in both the pooled ractopamine sample and when the densitometry values obtained from each individual animal were averaged ($P < 0.05$), relative to the mRNA levels in untreated longissimus muscle (Figure 2B). Interestingly, CALM1 expression increased in three of the four pigs fed ractopamine; it is unclear why one individual did not respond to the treatment (lane 6 in Figures 2A and 2B). These results, together with the fact that six cDNA clones had high homology to CALM1 mRNA transcripts in both the human and the rat (Table 1), suggest that CALM1 may be regulated pre-translationally by ractopamine and that the encoded protein product may play an important role in ractopamine-induced skeletal muscle hypertrophy and fiber type plasticity.

Calmodulin (CaM) is a ubiquitously expressed calcium-modulating protein and has been implicated in both skeletal muscle hypertrophy and MHC isoform transitions through the activation and regulation of the Ca^{+}/CaM-dependent protein phosphatase, calcineurin (CaN), and Ca^{+}/CaM-dependent kinases (CaMKs) (Naya

et al., 2000; Olson & Williams, 2000b; Semsarian et al., 1999). In C2C12 mouse myotubes, activated (Ca^+/CaM-bound) CaN and CaMKII are required for 2A MHC promoter activity in response to calcium ionophore treatment (Allen & Leinwand, 2002). Activated CaN and CaMKII regulate two families of transcription factors, myocyte-specific enhancer factor 2 (MEF2) and nuclear factor/activator of T cells (NFAT), both of which are necessary to increase 2A MHC promoter activity (Allen & Leinwand, 2002; Allen et al., 2001). Additionally, CaN induces gene expression during skeletal muscle hypertrophy through the promotion of NFAT interactions with the transcription factor GATA2 at promoter sequences (Olson & Williams, 2000a). Ca^+/CaM-dependent kinases also provide transcriptional control of gene expression (Corcoran & Means, 2001; Shimomura et al., 1996). Histone deacetylases (HDACs) interact with MEF2 to silence gene expression by histone condensation. Ca^+/CaM-dependent kinases phosphorylate serine residues in HDAC-4 and HDAC-5, which disrupts the MEF2:HDAC complex and restores gene expression (McKinsey et al., 2000; Olson & Williams, 2000a). Taken together with the novel finding that ractopamine stimulates the accumulation of CALM1 mRNA (this study), we hypothesize that β-adrenergic agonists transcriptionally activate CaM, which, in the presence of increased calcium flux, positively augments CaN and CaMK activity and muscle specific gene expression.

In porcine skeletal muscle, this may explain, in part, the changes in fiber type and contractile gene expression documented after ractopamine stimulation. The study conducted by Depreux et al. (2002) certainly supports this model, as the 2A MHC isoform was the only MHC isoform to increase in relative abundance after

ractopamine treatment for 3 d. This affect was lost after prolonged treatment, and 2B MHC became the predominant isoform expressed in skeletal muscle. Interestingly, CALM1 is also differentially expressed in mouse quadriceps relative to the soleus muscle (Campbell et al., 2001), which suggests a role for CaM in the maintenance of the fast twitch fiber phenotype. As high doses of ractopamine promotes the transition in MHC isoforms from 2A → 2AX → 2B in a time-dependent manner (Depreux et al., 2002), it is likely that CALM1 expression remains elevated in response to β-adrenergic stimulation in order to maintain fiber type plasticity.

Prolonged elevations in CALM1 expression may also be involved in the transcriptional control of muscle hypertrophy. Skeletal α-actin mRNA abundance in pigs (Grant et al., 1993; Helferich et al., 1990; Liu et al., 1994) and steers (Smith et al., 1989) increases in response to ractopamine without a general increase in total muscle mRNA. Importantly, the porcine skeletal α-actin promoter contains a MEF2 DNA element (Reecy et al., 1996), as do many muscle specific genes (Brand, 1997). As it now appears that CALM1 expression precedes changes in skeletal muscle phenotype after β-adrenergic stimulation (this study), CaMK activity and the regulation of MEF2:HDAC complexes at the skeletal α-actin promoter in response to ractopamine may also provide more mechanistic detail as to how β-adrenergic agonists stimulate skeletal muscle hypertrophy at the molecular level.

While transcriptional regulation by CaM-dependent mechanisms is certainly interesting and may explain the nuclear regulation of cellular phenotype in response to β-adrenergic agonists, the ubiquitous nature of calmodulin should not be

overlooked in future studies. In addition to CaN and CaMKs, CaM may also regulate the function of cellular proteins expressed in skeletal muscle. Calmodulin also has affinity for, and regulates the function of, the ryanodine-receptor (RyR), the inositol 1,4,5-trisphosphate type 1 receptor ($InsP_3R$), myosin light chain kinase (MLCK), plasma membrane Ca-ATPase, nitric oxide synthase (NOS), and adenylyl cyclases (Maier & Bers, 2002). Clearly, a ractopamine-induced induction of CALM1 expression to the magnitude (2-fold) suggested in this study may have far reaching cellular consequences.

The maintenance of calcium homeostasis within the muscle cell is vital to function. Multiple stimuli may converge on the ryanodine and dihydropyridine receptors to regulate calcium release from the sarcoplasmic reticulum (Wrzosek, 2000). As apocalmodulin (calcium-free CaM) has been demonstrated to be an effective agonist of skeletal muscle RyR function (Fruen et al., 2003; Tang et al., 2002), calmodulin-1 expression may stimulate intracellular calcium release in ractopamine-stimulated skeletal muscle, independent of increased calcium flux, to maintain or restore intracellular calcium homeostasis.

Implications

This is the first study to investigate differential gene expression in skeletal muscle after feeding ractopamine to pigs. After three days of treatment, we identified and confirmed the expression of the EF-hand calcium binding protein, calmodulin. Further analysis of the expression profile of calmodulin at multiple post-treatment time points, as well as the post-translational role of the encoded protein,

should further define the nature and role of elevated calmodulin expression in response to ractopamine. Nonetheless, this is the first study to indirectly implicate calcium-modulating proteins as potentially critical participants in signal transduction networks and cellular homeostasis in response to ractopamine in porcine skeletal muscle.

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Figures and Tables

Figure 1. Analysis of amplified subtraction products by gel electrophoresis. Amplicons generated after 25 cycles of PCR from subtracted (lane 1) and unsubtracted (lane 2) cDNA libraries as described in Materials and Methods. CDNA fragment size ranged from 1500 to 210 bp after subtraction suppressive hybridization.

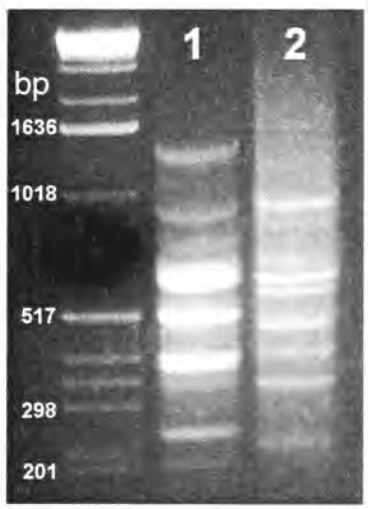


Table 1. Differentially expressed genes in porcine longissimus dorsi muscle after 3 days of ractopamine treatment as identified by suppression subtractive hybridization and BLAST analysis.

Putative Identification [a]	Accession No. [b]	Homology (bp) [c]
Calcium-modulating proteins		
Calmodulin-1 (phosphorylase kinase,	BC047523	90% (274/304)- Human
	U16850- (3X)	90% (131/144)- Human
	NM 031969	87% (191/219)- Rat
	AF178845	86% (204/235)- Rat
Homer homolog 1 (Drosophila)	AF093262	95% (396/385)- Human
Signal Transduction/ Chaperon Proteins		
Similar to protein phosphatase 1,	XM 018216- (4X)	88% (289/327)- Human
Heat shock 70kDa protein 9B (mortalin-2)	BC024034	90% (546/601)- Human
Similar to DnaJ (Hsp40) homolog,	BC033159- (3X)	86% (143/165)- Human
mRNA Processing Proteins		
Mago-nashi homolog, proliferation-	AF067173	94% (290/308)- Human
Heterogeneous nuclear ribonucleoprotein	BC007392	91% (142/155)- Human
Structural Proteins		
Syntrophin, beta 1	NM 021021- (2X)	88% (127/143)- Human
Synemin, isoform B	AJ310522	82% (106/128)- Human
Expressed Sequence Tags		
384525 MARC 2PIG Sus scrofa cDNA 5'	BI359811 [d]	99% (339/341)- Pig

[a] Putative identity was assigned by sequence homology after BLAST analysis. Two cDNA clones could not be identified due to poor sequence reaction results.
[b] GenBank Accession number. Multiple clones (number indicated in parenthesis) with homology to indicated GenBank entry.
[c] Percent homology of sequenced cDNA clone to GenBank entry. The number of matching base pair identities and species is indicated.
[d] Library constructed from pooled tissue from testis, ovary, endometrium, hypothalamus, pituitary, and placenta. Included in Table 2.

Table 2. Porcine-specific tentative consensus (TC) and singleton expressed sequence tag (EST) sequence identity assigned to differentially expressed cDNAs.

Putative Identification [a]	Homology (bp) [b]	Accession No. [c]	EST Name [d]	Tissue Source [e]	TC Identity [f]
Calmodulin-1	99% (338/339)	AW620097	861	Day 12 embryo	Singleton
	99% (485/486)	BM194704	EST329	Muscle	TC 66852
	88% (105/118)	BQ598252	MI-P-E3-agr-f-09-1-UM.s1	Day 45 fetus	TC 66012
	100% (216/216)	CB478983	Jns44_C04.f	Lymphoid	Singleton
Homer-1	-	-	-	-	-
Phosphatase Inhibitor 2	-	-	-	-	-
Mortalin-2	99% (223/225)	BQ600657	MI-P-E7-agz-g-08-1-UM.s1	Day 12 Conceptus	TC 66466
DnaJ	99% (344/345)	BF712138	MI-P-E4-abd-a-12-1-UM.s1	Day 14 Embryo	TC 67651
Mago-nashi	98% (417/425)	BF708768	MI-P-AYO-nai-g-09-0-UI.s1	Placenta	TC 73859
HNRPD-like protein	100% (192/192)	BU946720	PS24U-210	Skin	*NA*
Syntrophin	99% (298/300)	C94948	C94948	Back fat	TC78258
Synemin	-	-	-	-	-
EST	99% (339/341)	BI359811	384525	Pooled	TC 76532

[a] Putative identity was assigned by sequence homology after BLAST analysis. Refer to Table 1.
[b] Percent homology of sequenced cDNA clone to GenBank entry.
[c] GenBank Accession number.
[d] Identity of EST sequence.
[e] Tissue from which EST was generated.
[f] Tentative porcine consensus sequence identifier. TIGR Porcine Gene Index (SsGI), Version 6.0, Release Date - April 30, 2003. http://www.tigr.org/tdb/tgi/ssgi/

Figure 2A. Northern blot analysis to confirm differential expression of calmodulin-1 (CALM1) in longissimus dorsi muscle samples from pigs fed two levels of Paylean (0 ppm or 60 ppm) for 3 days. Autoradiographs of a Northern blot probed for CALM1 (upper) and β-Actin (lower) steady state mRNA as described in the Materials and Methods section. Lanes 1-4: individual animals fed 0 ppm Paylean; Lanes 5-8: individual animals fed 60 ppm Paylean; Lane 9: pooled RNA samples from pigs fed 0 ppm Paylean (-); Lane 10: pooled RNA samples from pigs fed 60 ppm Paylean (+). Twenty milligrams of total RNA was loaded in each lane. *N*=4 pigs per treatment.

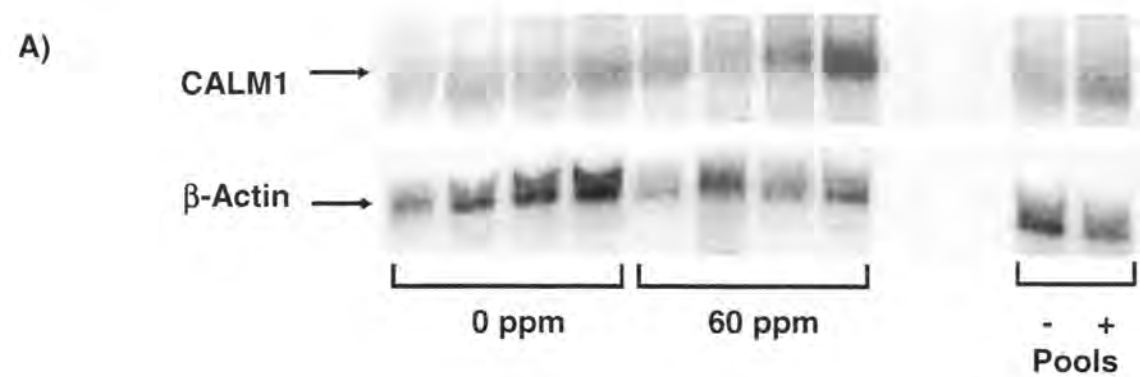
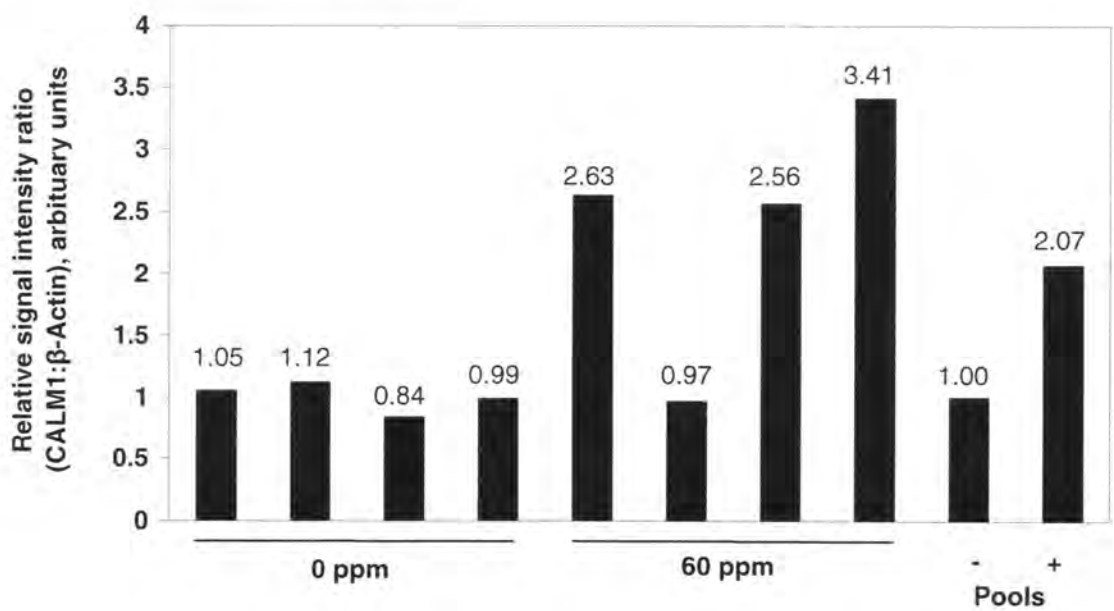


Figure 2B. Graphical summary of Northern analysis to confirm differential expression of CALM1 in longissimus dorsi muscle samples from pigs fed two levels of Paylean (0 ppm or 60 ppm) for 3 days (Figure 2A). Values are the signal intensity ratio of CALM1:β-Actin steady-state mRNA relative to the 0 ppm pooled RNA values. Fold change from base line (0 ppm) is indicated.



CHAPTER 4. GENERAL CONCLUSIONS

The livestock industry has exploited the anabolic enhancing capacity of growth promotants, such as antimicrobials, growth factor analogues, and β-adrenergic agonists, to improve production efficiency and output of nutritious meat products. In particular, β-adrenergic agonists improve lean carcass composition, dressing percentage, growth rate, and growth efficiency without adversely affecting meat quality in most species. One synthetic β-adrenergic agonist, ractopamine, has a profound effect on skeletal muscle mass in the pig. However, sufficient detail as to how ractopamine directly modifies skeletal muscle phenotype has remained elusive.

The further elucidation of ractopamine's mode of action in skeletal muscle may enhance the utility of future growth promotants in livestock production systems. For example, if ractopamine does indeed utilize a unique molecular mechanism to direct muscle growth, the identification of that mechanism would be beneficial to the development of targeted growth promotants that augment the pathway more effectively. The β-adrenergic receptor is subject to constant regulation by both desensitization and transcriptional down-regulation. If downstream signal transduction proteins involved in the transmission of this stimulus (ractopamine) are identified, it is conceivable that pharmacological agonists could be utilized to activate these proteins independent of the β-adrenergic receptor. Such an approach would effectively by-pass the cell's intrinsic ability to desensitize the β-adrenergic receptor, which may lead to a constitutively activated system and further enhancement in the rate of protein accretion and muscle hypertrophy.

Alternatively, the identification of the critical proteins involved in the intracellular signal transduction pathway(s) activated by β-adrenergic agonists, and the selective activation of these proteins, could benefit the field of human medicine, particularly in the prevention and/or treatment of cachexia and muscular dystrophies. As β-adrenergic stimulation is closely coupled with cardiac contractility and hypertrophy, potential cardiovascular complications preclude the use β-adrenergic agonists to spare skeletal muscle mass from severe atrophy and loss of function in a clinical setting. Hypothetically, if the molecular mechanisms that underlie skeletal muscle hypertrophy in response to β-adrenergic agonists are distinct from those present in cardiac muscle, effective pharmaceutical intervention and/or gene therapy becomes reasonable. This is especially true if the difference in the two pathways is subtle. For example, the hypertrophic response in skeletal muscle may require one protein isoform whereas the cardiac-specific pathway may utilize a splice variant of that same protein or an isoform encoded by a different gene. In the post-genomic era, our ability to systematically dissect the biochemical and molecular pathways that trigger physiological responses is greatly enhanced, which allows for exploration of questions that were once thought to be unanswerable.

This project investigated the effect of ractopamine on gene regulation in skeletal muscle in the pig. In the first study, the hypothesis that ractopamine directly regulates the expression of the porcine skeletal α-actin gene via cis-acting DNA elements was tested. β-Adrenergic agonists stimulate the accumulation of skeletal α-actin mRNA without a global augmentation of total RNA synthesis. This increase

in pre-translational expression parallels increases in myofibrillar protein expression and skeletal muscle hypertrophy in porcine skeletal muscle that has been stimulated with ractopamine. Therefore, identifying regulatory mechanisms that control the expression of this specific gene should provide insight into the mechanisms by which ractopamine induces skeletal muscle hypertrophy.

We developed an *in vitro* model that was sufficient to recapitulate the increased pre-translational expression of skeletal α-actin to test our hypothesis. However, we were unable to identify a ractopamine-responsive DNA regulatory element within the full-length porcine skeletal α-actin promoter. From our data, we concluded that either another regulatory mechanism within the porcine skeletal α-actin locus contributes to the transcriptional regulation of this gene, or that mRNA stability is in some way altered after ractopamine-stimulation. Both mechanisms may be valid explanations for the observed increase in skeletal α-actin mRNA abundance in pigs treated with ractopamine and warrant empirical investigation.

In a second study, we identified differentially expressed genes by suppression subtractive hybridization. With this technique, a cDNA library is normalized and differentially expressed genes are enriched and identified. Presumably, these genes are regulated by ractopamine and/or are required for sustained muscle hypertrophy and fiber type plasticity.

We identified nine genes and one expressed sequence tag that are possibly regulated in response to ractopamine in porcine skeletal muscle. This work revealed that ractopamine positively augments the expression of calmodulin-1 and is the first such study to indirectly implicate calcium-modulating proteins as potentially critical

participants in signal transduction networks and/or cellular homeostasis in response to β-adrenergic agonists. Calcium, calmodulin, and calmodulin-dependent proteins have been demonstrated to be potent regulators of skeletal muscle hypertrophy and muscle fiber phenotype. Further analysis of the expression profile of both CALM1 and homer-1b at multiple post-treatment time points should further define the nature of their expression in response to β-adrenergic agonists.

In summary, the research described herein has provided insight into the molecular events that underlie skeletal muscle adaptation to β-adrenergic stimulation. In no way has this been an all-inclusive study, but it has underscored the complexity of protein-protein interactions that convey responsiveness to ractopamine within the myofiber. Certainly, increased production of cyclic adenosine monophosphate (cAMP) is a well documented cellular response to β-adrenergic receptor activation, but other second messengers, such as calcium, may be more intimately involved in the regulation of gene expression and skeletal muscle hypertrophy. Future work must use molecular techniques beyond the simplistic measure of cellular cAMP concentration to delineate the intracellular signal transduction pathways that mediate skeletal muscle adaptation to β-adrenergic agonists.

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Ability is what you're capable of doing. Motivation determines what you do. Attitude determines how well you do it." – Lou Holtz

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